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TELOMERE BIOLOGY IN METAZOA

NUNO M. V. GOMES

DOUTORAMENTO EM ENGENHARIA BIOMÉDICA E BIOFÍSICA

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DOUTORAMENTO EM ENGENHARIA BIOMÉDICA E BIOFÍSICA

2011

DEDICATION

Dedicated to my wonderful family
for brightening every day of my life.

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TELOMERE BIOLOGY IN METAZOA

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ABSTRACT

Telomerase, the enzyme that maintains telomeres, is absent from most adult human somatic cells, producing a progressive telomere shortening that limits the proliferative potential of primary human cell cultures (Shay and Wright 2007). This programmed telomere shortening, replicative aging, functions as a tumor suppressor program that generates a barrier for the outgrowth of tumors. Remarkably, this telomere tumor suppressor program is not conserved in laboratory rats and mice, which have long telomeres and constitutive telomerase (Sherr and DePinho 2000; Wright and Shay 2000). The present study examines over 60 mammalian species to determine the phylogenetic distribution of the telomere tumor suppressor pathway. Phylogeny based statistical analysis demonstrates that telomere length inversely correlates with lifespan but not body size, while telomerase expression inversely correlates with body size but not lifespan. The ancestral mammalian phenotype was determined to have short telomeres and

repressed telomerase. At least 5-7 independent times in different orders smaller, shorter lived species changed to having long telomeres and expressing telomerase, suggesting tradeoffs between the advantages and drawbacks of using replicative aging as a tumor suppression mechanism. We show that one advantage is consistent with reducing the energetic/cellular costs of specific oxidative protection mechanism needed to maintain short telomeres. We propose that the telomere tumor suppressor pathway represents an initial adaptation to the increased mutational load of homeothermy by ancestral mammals, has adaptive advantage in large and long-lived animals, but has been abandoned by many species. These observations resolve a longstanding confusion about the use of telomeres in humans and mice, support a role for telomere length in limiting lifespan, provide a critical framework for interpreting studies of the role of oxidative protection in the biology of aging, and identify which mammals can be used as appropriate model organisms for the study of the role of telomeres in human cancer and aging.

keywords: evolution. telomeres, telomerase, senescence, mammals

RESUMO

As células somáticas humanas normais exibem uma capacidade proliferativa limitada, um fenómeno conhecido como “limite de Hayflick”. As células fetais dividem-se mais vezes em cultura do que as de uma criança, que por sua vez se dividem mais do que as de um adulto. Os telómeros são os relógios moleculares que permitem às células contarem o seu número de divisões. Os telómeros são as sequências repetitivas de ADN encontradas nos extremos dos cromossomas lineares. Cada um dos 92 telómeros humanos contém milhares de repetições da sequência de seis nucleótidos TTAGGG e as proteínas associadas aos telómeros. O comprimento dos telómeros diminui quer em função da idade dos tecidos do dador, quer com o número de divisões celulares em cultura.

A telomerase é uma ribonucleoproteina celular transcriptase reversa que utiliza o seu componente catalítico (hTERT) para sintetizar ADN telomérico (TTAGGG)n directamente nas extremidades dos cromossomas. Em humanos, esta enzima é expressa em tecidos embrionários e em células germinais específicas, mas não é detectada na maioria das células somáticas normais, o que conduz a um encurtamento progressivo dos telómeros que limita o potencial proliferativo das células primárias humanas.

Este encurtamento programado dos telómeros - senescência replicativa – funciona como um programa supressor tumoral que gera uma barreira contra o sobrecrescimento tumoral (85% dos tumores humanos possuem actividade da enzima telomerase e são capazes de manter os seus telómeros). Notavelmente, este programa supressor tumoral telomérico não se encontra preservado nas ratazanas e nos ratos de laboratório, que têm

telómeros longos e telomerase constitutiva. O estudo presente examina mais de 60 espécies de mamíferos de modo a determinar a distribuição filogenética deste mecanismo supressor tumoral.

A expressão de telomerase em culturas de fibroblastos em divisão provenientes de dadores adultos foi usada para determinar a força da repressão da telomerase em células somáticas mesenquimatosas. As células cultivadas em condições não ideais (ex: falta de um micronutriente, oxigénio a 20%), exibem frequentemente uma paragem de divisão chamada estase ("STASIS"- stress or aberrante signaling induced senescence), que é independente do encurtamento telomérico. A presença de estase cedo (dentro de 15 duplicações) também forneceu um fenótipo adicional.

Neste estudo examinei a expressão da telomerase, o comprimento dos telómeros, o peso corporal e a longevidade. Usando o resultado da análise de modelos de regressão que levam em linha de conta a ascendência comum, prevista pela filogenia dentro de uma matriz filogenética dos quadrados mínimos (PGLS) verifica-se que a expressão da telomerase se correlaciona de modo significativo com o inverso da massa corporal ($p=0.0082$), mas não apresenta efeitos independentes com a longevidade ($p=0.34$). A mesma análise demonstrou que o comprimento dos telómeros apresenta uma significativa correlação negativa com a longevidade ($p=0.0032$) acima do previsto pela massa corporal por si só, mas não se observou uma associação independente entre o comprimento dos telómeros e a massa corporal ($p=0.71$).

Um controlo rigoroso da senescência replicativa como mecanismo de supressão tumoral requer telómeros curtos juntamente com repressão da telomerase. Contudo, estes

resultados sugerem que um decréscimo dos níveis de expressão da telomerase pode, por si só, conferir vantagens à medida que o número de células do corpo aumenta (com o tamanho). Isto pode dever-se à capacidade da telomerase para reparar telómeros que sofreram eventos de deleção "catastróficos" (por exemplo, a expressão da telomerase pode permitir que uma célula pré-maligna em que se encontrem ausentes pontos de controlo celular sobreviva a deleções resultantes de paragens dos garfos de replicação ao nível dos telómeros). Por outro lado, a expressão da telomerase pode ter efeitos adicionais independentes da manutenção dos telómeros. Existe uma correlação bem estabelecida entre a massa corporal e a longevidade. Quando o número de células atinge um certo patamar, a associação independente entre telómeros curtos com um aumento da longevidade sugere que o estabelecimento completo da senescência replicativa é necessário de modo a suprimir a formação tumoral durante períodos de tempo mais prolongados. Observações anteriores levaram a concluir que a expressão da telomerase *in vivo* decresce com o aumento da massa corporal em roedores, mas não foi observada uma relação com a longevidade quer para a actividade da telomerase, quer para o comprimento telomérico. Os resultados deste projecto demonstram que, numa análise global da classe dos mamíferos, o comprimento telomérico coevolui com a longevidade.

O comprimento telomérico ancestral na base dos mamíferos placentários foi reconstruído usando modelos de estimação de probabilidade máxima que geram o valor mais provável na raiz de uma determinada árvore filogenética sob o modelo evolutivo de movimento Browniano juntamente com o parâmetro "lambda", que mede a força do sinal filogenético. O estado ancestral determinado foi de 18.6 kb com um lambda igual a 1,

indicando um signal filogenético muito forte. O estado ancestral de repressão da telomerase foi estimado usando uma matriz de transição probabilística (Markov). A probabilidade de o mamífero placentário ancestral reprimir a telomerase foi calculada como sendo alta (1), em comparação com a probabilidade de a telomerase ter sido expressa (0). Estes resultados permaneceram qualitativamente iguais mesmo se cada ordem for analisada separadamente. A frequência de transição de expressão para repressão foi estimada como sendo perto de zero, o que significa que apenas ocorreram transições de repressão para expressão. Apesar de haver apenas um número limitado de estudos, um extenso grupo de espécies aquáticas poiquilotérmicas (de equinodermes a peixes cartilagíneos ou ósseos) possuem telómeros curtos e expressam a telomerase em muitos dos seus tecidos. No entanto, conseguimos determinar que é provável que o fenótipo ancestral dos mamíferos placentários ancestrais consistisse em ter telómeros curtos e reprimir a enzima telomerase. Isto sugere que uma das primeiras adaptações à homeotermia, (com o aumento da carga mutacional que a acompanha), foi a repressão da telomerase nas células somáticas adultas que possuíam já os telómeros curtos, levando assim ao início do encurtamento telomérico como um mecanismo de protecção tumoral.

O mamífero ancestral foi provavelmente mais semelhante aos mamíferos não placentários, mas não foi possível esclarecer por completo o seu fenótipo telomérico. A sequência TTAGGG não possui sítios de restrição. O comprimento telomérico é normalmente determinado pela digestão do ADN genómico com uma mistura de quatro enzimas de restricção reconhecedoras de quatro bases de modo a remover ADN de sequência diversa do lado telomérico interno (centromérico), sendo o tamanho dos

telómeros medido em géis de agarose. Os mamíferos não placentários possuem telómeros em que longas extensões de repetições teloméricas foram interrompidas por ADN contendo sítios de restrição. O comprimento dos telómeros de wombats e coalas parece ser menor que 2 kb quando digerido com a nossa mistura de rotina de seis enzimas, mas exibem padrões completamente diferentes quando digeridos com enzimas individuais. O comprimento dos telómeros variou de longo a muito curto dependendo de qual enzima de restrição reconhecedora de quatro bases fosse utilizada para digerir o ADN, não tendo assim sido possível determinar o comprimento telomérico. A natureza das sequências intratelomérica encontradas nos mamíferos não placentários e se estas reflectem acontecimentos passados de recombinação/inserção ou um processo corrente envolvido na manutenção dos telómeros ainda não foi determinado. Contudo, a ausência de expressão da telomerase por células de coala, a sua paragem de crescimento em cultura após apenas 38 divisões, mesmo após o bloqueio da função de outras barreiras do ciclo celular, e a sua subsequente imortalização após a introdução de hTERT, sugere que pelo menos um marsupial utiliza a senescência replicativa e que apenas a porção mais terminal da sequência telomérica não interrompida se encontra a funcionar neste processo.

Acredita-se que as espécies ancestrais de mamíferos eram pequenas. Contudo a maioria das pequenas espécies actuais (com menos de 1 kg) possuem telómeros longos e expressam a telomerase. A análise filogenética sugere que o fenótipo ancestral dos mamíferos consistia em ter telómeros curtos e reprimir a telomerase como uma adaptação inicial à homeotermia, e que a aquisição de telómeros longos conjuntamente com a ausência de repressão da telomerase representam alterações secundárias que

proporcionaram vantagens adaptativas a espécies que, ou permaneceram pequenas ou que evoluíram de precursores maiores (tal como se pensa ter ocorrido em morcegos). Estas espécies mais pequenas adquiriram telómeros mais longos e expressão de telomerase pelo menos 5-7 vezes e de modo independente durante a evolução. Uma vantagem pode reflectir um compromisso entre os benefícios da supressão tumoral e os custos de limitar a regeneração. Doenças humanas envolvendo mutações na telomerase causam esgotamento prematuro das células estaminais e uma variedade de doenças associadas com a idade tais como insuficiência esporádica da medula óssea, disqueratose congénita e fibrose pulmonar idiopática, levantando a hipótese de que o encurtamento telomérico pode contribuir para alguns aspectos do envelhecimento humano. Uma vantagem da não utilização da senescência replicativa seria um aumento da capacidade regenerativa. Um compromisso adicional pode ser um investimento mais elevado de recursos destinados à protecção contra o dano oxidativo em espécies com telómeros curtos. Os radicais livres danificam preferencialmente a sequência GGG, que nos mamíferos se encontra presente a cada seis pb nas repetições teloméricas TTAGGG. Além disso, uma propriedade fundamental dos telómeros (a supressão local de indicadores de lesões do ADN de modo a que os extremos de cromossomas lineares não sejam reconhecidos como quebras de cadeia dupla) leva a que lesões teloméricas oxidativas sejam reparadas de um modo muito mais lento do que no restante genoma. Este facto, aumenta a probabilidade de que quebras de cadeias simples sejam convertidas em quebras de cadeia dupla e a consequente perda de sequências teloméricas. Telómeros muito longos (demasiado longos para contar as divisões celulares de modo eficiente), permitem contudo que

grandes segmentos teloméricos sejam perdidos sem que seja comprometida a divisão celular. De modo semelhante, a expressão da telomerase poderia permitir a reparação e o alongamento de telómeros com encurtamentos abruptos. A manutenção de telómeros suficientemente curtos para limitar a capacidade proliferativa das células e funcionar como um mecanismo supressor tumoral pode levar a uma necessidade acrescida de investir recursos em mecanismos de protecção oxidativa. Para examinar esta hipótese de trabalho, determinámos a sensibilidade de 15 espécies a dois tipos de stresse oxidativo, tert-Butilhidroperóxido e arseniato de sódio. A relação entre a sensibilidade e o comprimento telomérico como variável independente da massa corporal/longevidade foi significativa usando análise de contraste independente da filogenia para ambos os agentes (tert-Butilhidroperóxido p=0.032; e arseniato de sódio p=0.017). Esta relação também se manteve considerando quer a longevidade, quer a massa como variáveis independentes. A resposta ao arseniato de sódio é mais dramática que ao tert-Butilhidroperóxido. As espécies podem ser agrupadas em dois grupos cuja sensibilidade difere aproximadamente 6 vezes sem que as espécies desta análise limitada exibam valores intermédios. A resistência ao arseniato de sódio foi independente da capacidade das células crescerem bem sob as condições normais de cultura, dado que duas espécies com telómeros curtos que exibiram estase (a baleia cinzenta e o tapir) ainda apresentaram o fenótipo de resistência. Apesar do mecanismo para o diferente comportamento do tert-Butilhidroperóxido e do arseniato de sódio ser desconhecido, pode reflectir compartimentalização celular ou diferentes propensões a lesar lípidos, proteínas ou ADN.

Há muito que se colocou a hipótese de as lesões oxidativas serem um dos principais determinantes da longevidade. Os resultados presentes mostrando uma relação de dependência entre o comprimento dos telómeros e o arseniato de sódio / tert-Butilhidroperóxido demonstram que irá ser importante considerar o comprimento telomérico como uma variável independente ao comparar o nível de resistência a diferentes stresses oxidativos como uma função da longevidade.

Propõe-se assim que o mecanismo supressor tumoral telomérico representa uma adaptação inicial dos mamíferos ancestrais ao aumento de mutações associado à homeotermia tem vantagens adaptativas em animais grandes / de maior longevidade, mas foi abandonado por muitas espécies. Estas observações resolvem uma longa confusão acerca do uso dos telómeros em humanos e ratos, definem uma importante característica distintiva das ordens filogenéticas em mamíferos, fornecem um cenário crítico para a interpretação de estudos do papel da protecção oxidativa na biologia do envelhecimento, e identificam que mamíferos podem ser usados como organismo modelo apropriado para o estudo do papel dos telómeros no cancro e envelhecimento humanos.

Palavras chave: evolução, telómeros, telomerase, senescência, mamíferos

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a-MEM	Minimum Essential Medium *(*MEM*) Alpha* Medium
ALT	Alternative Lengthening of Telomeres
At-TERT	*Arabidopsis thaliana* Telomerase Reverse Transcriptase (Protein Component)
BJ	Human Foreskin Fibroblast Cell line
bp	Base Pair(s)
BSA	Bovine Serum Albumin
CDK4	Cyclin-dependent Kinase 4
chTERT	Chicken Telomerase Reverse Transcriptase
DAPI	4', 6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ds	Double-stranded
FBS	Fetal Bovine Serum
FGM	Clonetics Fibroblast Growth Medium
FIGE	Field Inversion Gel Electrophoresis
FISH	Fluorescence *in situ* Hybridization
FITC	Fluorescein Isothiocyanate
FLARE	Fragment Length Analysis using Repair Enzymes
fmol	Femtomole

Fpg	*E. coli* Formamidopyrimidine-DNA Glycosylase (Fpg)
fTERT	*Fugo* Telomerase Reverse Transcriptase (Protein Component)
Hpv	Human Papilloma Virus
hTERT	Human Telomerase Reverse Transcriptase (Protein Component)
hTR/ hTERC	Human Telomerase RNA (template RNA Component)
mTR	Mouse Telomerase RNA (template RNA Component)
ITAS	Internal Telomerase Assay (TRAP) Standard
kb	Kilobase Pair(s)
LD90	Lethal dose that Kills 90% of the cells
M1	Mortality Stage 1
M2	Mortality Stage 2
Mb	Megabase Pair(s)
MEFS	Mouse Embryo Fibroblasts
mRNA	Messenger RNA
mTERT	Mouse Telomerase Reverse Transcriptase (Protein Component)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Population Doublings
RLgT	Retrovirus expressing SV40 LgT
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Sc	*Saccharomyces cerevisiae*

SCGE	Single Cell Gel Electrophoresis
SE (SEM)	Standard Error
Sp	*Schizosaccharomyces pombe*
STASIS	STress or Aberrant Signaling Induced Senescence
SV40	Simian Virus 40
TAS	Non-coding Sub-telomere region of Plasmodium falciparum
tBH	tert-Butyl hydroperoxide
TPE	Telomere Position Effects
TRAP	Telomeric Repeat Amplification Protocol
TRF	Telomere Restriction Fragment
xTERT	*Xenopus* Telomerase Reverse Transcriptase (Protein Component)

CHAPTER ONE

General Introduction and Literature Review

1. Summary

Telomere-based replicative senescence is thought to function as a potent mechanism of tumor protection in humans. Whether this mechanism is conserved in other species is still unclear. In this general introduction I present an inter-species critical overview of some of the available literature on the fundamental biology of telomeres and telomerase during development, regeneration, cancer and aging of living organisms during their evolutionary journey through time.

2. Introduction

Telomeres are the repetitive DNA sequences found at the ends of linear chromosomes (Muller 1938; McClintock 1941). Each of the 92 human telomere ends is formed by thousand of repeats of the six nucleotide sequence TTAGGG bound by telomere-associated proteins such as the shelterin complex (Blackburn and Gall 1978; Moyzis, Buckingham et al. 1988; deLange 2005) (Fig 1.1).

During DNA replication the leading strand of linear chromosomes is synthesized as a continuous molecule that can potentially replicate all the way to the end of a linear template. The lagging strand is made as a discontinuous set of short Okazaki fragments,

each requiring a new RNA primer to be laid down on the template that are then ligated to make a continuous strand. As there is no DNA beyond the end for a priming event to fill the gap between the last Okazaki fragment and the terminus, the lagging strand cannot replicate all the way to the end of a linear chromosome. This leaves a 3' overhang that cannot be filled, and this has been called the "end replication problem" (Watson 1972; Olovnikov 1973). The leading strands are also processed to leave a 3'overhang (Wright and Shay 2000). Since one strand cannot replicate its end, telomere shortening will occur, and once inherited by the daughter cells, the process repeats itself in subsequent divisions (Olovnikov 1973). Human telomeres sizes range from ~15 kb at birth to sometimes less than 5 kb in chronic disease states (Shay and Wright 2004).

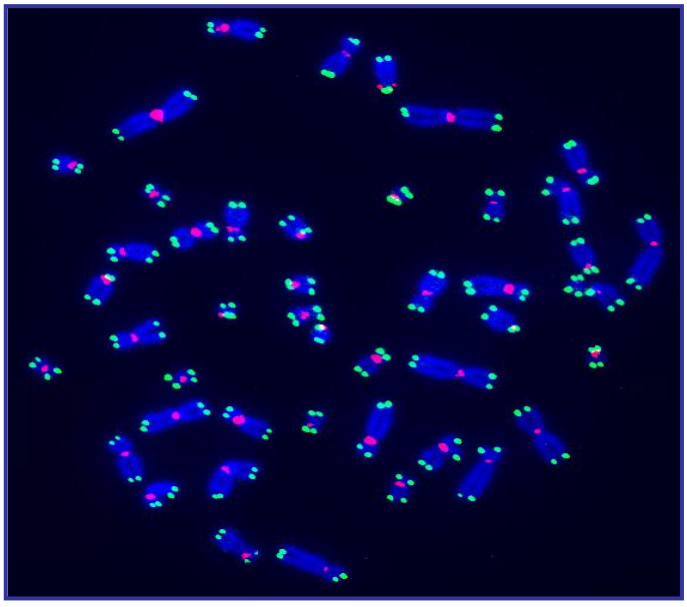


Fig. 1.1- A metaphase spread of a normal human male BJ fibroblast cell. Chromosomes are stained with DAPI and shown in blue. Telomeres are stained with a telomeric probe

and shown in Green. Centromeres are probed with a centromeric probe and shown in Red. (Courtesy of Ying Zou)

Normal human somatic cells display a limited capacity to proliferate, a phenomenon known as the “Hayflick limit” (Hayflick and Moorhead 1961). Fetal cells divide more times in culture than those from a child, which in turn, divide more than those from an adult. Telomeres provide the molecular clock that determines this replicative lifespan (Harley, Futcher et al. 1990). Human telomere length decreases both as a function of donor age in tissues and number of cell divisions in culture (Harley, Futcher et al. 1990; Hastie ND, Dempster M et al. 1990; Allsopp, Vaziri et al. 1992; Chang and Harley 1995). Replicative aging can be divided into 2 stages: Mortality stage 1 (M1 or Senescence) and Mortality stage 2 (M2 or Crisis). M1 occurs when most chromosomes still have several thousand base pairs of telomeric sequences left at their ends (Shay and Wright 2001). This stage is thought to be induced by DNA damage signals produced by one or a few particularly short telomere ends. DNA damage signaling from short telomeres, loss of the 3' G-rich telomere single-strand overhangs, and telomere position effects have all been suggested as potential inducers of M1. In the absence of cell-cycle checkpoint pathways (e.g. p53 and or p16/Rb), cells bypass M1 senescence and telomeres continue to shorten eventually resulting in M2/crisis (Shay and Wright 2001). M2 represents the result of multiple critically short telomeres when cells are no longer able to protect the ends of chromosomes so that end-to-end fusions occur,

leading to genomic instability and growth arrest or cell death. Rarely cells escape from M2 and become immortal almost universally due to the upregulation or reactivation of the enzyme telomerase, which is able to repair and maintain the telomeres. Senescent cells (due to telomere shortening as well as other inducers of irreversible growth arrest) can be stained by senescence associated β-galactosidase, and exhibit alterations in protein expression, such as increased secreted growth factors, cytokines, extracellular matrix, and degradative enzymes (Krtolica, Parrinello et al. 2001).

Telomerase is a ribonucleoprotein cellular reverse transcriptase that uses its catalytic component (hTERT) to synthesize telomeric DNA $(TTAGGG)_n$ directly onto chromosome ends (Feng J, Funk WD et al. 1995; Nakamura TM, Morin GB et al. 1997). The internal RNA component (hTR or hTERC) contains the template complementary to the telomeric single-strand overhang (Greider and Blackburn 1985; Morin 1989). After adding six bases, the enzyme pauses while it translocates the template RNA for the synthesis of the next 3' DNA repeat. This leads to additional rounds of replication of the 3'end of the G-rich strand (i.e. telomerase is a processive enzyme), thus compensating for telomeric losses due to the end replication problem and perhaps other end processing events (Shay and Wright 2001). In humans, this enzyme is expressed in embryonic tissues and specific germline cells. Telomerase is detected in fetal and adult testis but is neither found in most normal somatic cells, nor in non-dividing oocytes and mature spermatozoa(Shay and Wright 2004; Liu, Bailey et al. 2007). The exceptions are specific proliferative cells of renewal tissues (e.g. hematopoietic stem cells, activated

lymphocytes, basal cells of the epidermis, proliferative endometrium, and intestinal crypt cells) (Shay and Wright 2004). Many of these stem or stem-like cells in adult humans can activate telomerase activity when stimulated to divide. Low levels of telomerase activity may be sufficient to slow but not to prevent telomere shortening. Human intestine or skin telomeres shorten as a function of age although low levels of telomerase can be found in crypt cells and basal keratinocytes. In normal somatic cells and even in stem-like cells expressing telomerase, progressive telomere shortening occurs, eventually leading to senescence (Shay and Wright 2004). Introduction of the telomerase catalytic protein component (hTERT) into normal telomerase negative cells results is restoration of telomerase activity and telomere maintenance or elongation and immortalization (Bodnar, Ouellette et al. 1998). In some cell types in which the culture conditions are inadequate, it has been demonstrated that growth inhibitory genes can be activated due to a variety of environmental stresses in a process variously termed, premature senescence, culture shock, stress-induced senescence or STASIS (STress or Aberrant Signaling Induced Senescence) (Shay and Wright 2004). In cell culture if the conditions are inadequate, hTERT alone will not immortalize cells.

There are specific proteins (shelterin) associated with human telomeres. TTAGGG is recognized directly at least by the three shelterin subunits, TRF1, TRF2, and POT1. These are interconnected by at least three additional shelterin proteins, TIN2, TPP1, and Rap1, forming a structure that enables cells to distinguish telomeres from sites of DNA damage. Without TRF2, telomeres are no longer hidden from the DNA damage

surveillance and chromosome ends are inappropriately processed by the DNA repair machinery (deLange 2005). Shelterin is implicated in the formation of T-loops, first identified in human and mouse cells (Griffith, Comeau et al. 1999). The telomeric overhang has been proposed to invade the double-stranded telomeric DNA forming a lariat structure, base pairing with the C-strand and displacing the G-strand (Fig. 1.2). T-loops are a conserved aspect of telomere structure and have been speculated to protect telomeres and regulate telomerase (deLange 2005).

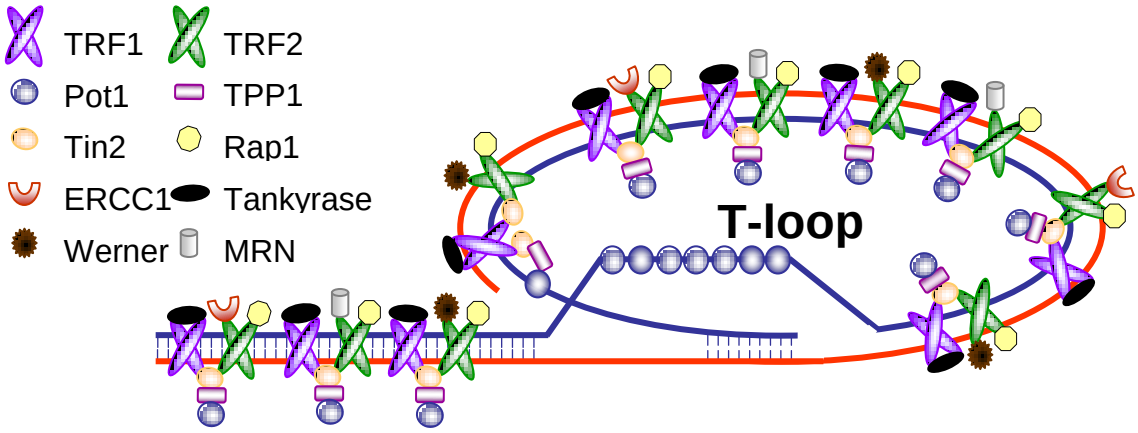


Fig. 1.2- The telomeric T-loop and associated protein complex. (Courtesy of Agnel Sfeir)

Telomere-based replicative senescence is thought to have evolved as a tumor protection mechanism in long-lived organisms such as humans, preventing the early development of cancer (Wright and Shay 2000). Normal human fibroblasts essentially

never immortalize in culture in part because at least three independent tumor prevention pathways (p53, p16INK4a/pRB, telomere shortening) have to be altered to allow immortal cell growth (Wright and Shay 2000). Cancer cells must acquire many mutations before they became malignant (Shay and Roninson 2004). Replicative aging blocks this progression by halting cell division before many mutations are able to accumulate within a single cell (Fig. 1.3). The cell containing an initial mutation must expand to a population size of perhaps one million cells before there is a reasonable probability for a second mutation to occur, so each mutation would require at least 20 divisions ($2^{20}=10^6$). Since most mutations are recessive, an additional clonal expansion is required to eliminate the remaining wild-type allele (usually through loss of heterozygosity). Limiting the number of available cell divisions to less than 100 would thus prevent pre-malignant cells from dividing after accumulating only a few mutations, and thus block their progression (Shay and Wright 2004). This hypothesis is supported by the finding that ~85% of human tumors have upregulated or reactivated telomerase activity and are able to maintain their telomeres Immortalization may occur by gene(s) mutation in the telomerase repression pathway (Tanaka H, Horikawa I et al. 2005).

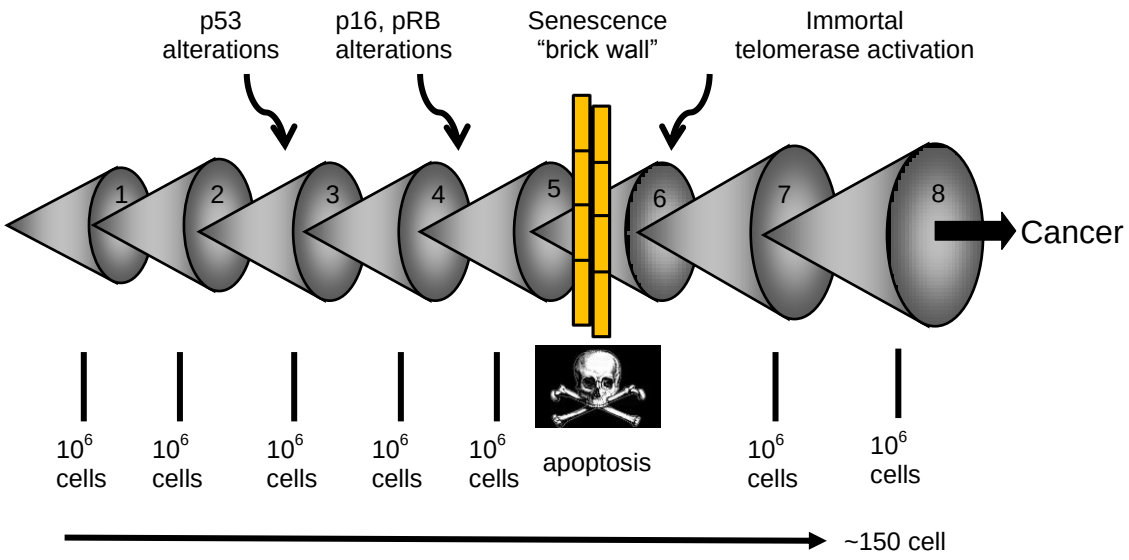


Fig. 1.3- Replicative aging and cancer. Multiple mutations are required before a cell can become malignant. This occurs as a series of clonal expansions. This uses a sufficient number of cell doublings so that senescence imposed by telomere shortening forms a barrier to the progression of tumor cells.

Another way telomeres can be maintained is through telomerase independent mechanisms known as alternative lengthening of telomeres (ALT) (Bryan, Englezou et al. 1997). This ALT pathway is only detected in a few rarer cancers (e.g. sarcomas), but is low in the more frequent epithelial neoplasias (carcinomas). This may reflect tighter telomerase regulation in mesenchymal versus epithelial tissues (Henson JD and RR. 2010). The ALT pathway is characterized by an array of phenotypes such as a very heterogeneous distribution of telomere sizes and length fluctuations, ALT-associated PML bodies (APBs), higher levels of telomere sister chromatic exchanges (T-SCE), and

raised levels of C-circles (Henson JD and RR. 2010). Recent studies in mice have suggested that telomerase-independent telomere elongation plays a role in normal development (Liu, Bailey et al. 2007). Mice oocyte telomere elongation following fertilization seems to be achieved through a recombination based mechanism characterized by extensive T-SCE. At the blastocyst stage, telomerase appears to take control of telomere maintenance (Liu, Bailey et al. 2007). Undifferentiated mouse ES cells expressing a gene cluster (Zscan4) undergo rapid telomere extension and long-term genomic stability, probably by telomere recombination or T-SCE. Unlike other cells that display T-SCE, such ALT tumor cells and survivors of telomerase knockout Terc2/2 ES cells, telomerase activity is detected in Zscan4 ES cells (Zalzman, Falco et al. 2010).

Telomeres are essential to prevent chromosome ends from being recognized as double-strand breaks. In addition, telomeres regulate cellular proliferation, survival, chromosome positioning, prevent DNA recombination, and participate in proper mitotic and meiotic divisions (Table 1.1) (Teixeira and Gilson 2005). As telomeres shorten during cellular aging there may be de-repression of genes near telomeres eventually leading to reactivation of other previously silenced genes. This process could occur on all or only in a subset of chromosome ends and is known as telomere position effects (TPE) (Baur, Zou et al. 2001). Telomere dysfunction has been implicated in a variety of human age related diseases (e.g. Werner syndrome) (Crabbe, Jauch et al. 2007). Mutations in telomerase genes have also been linked to some pathologies such as idiopathic pulmonary

fibrosis, aplastic anemia and dyskeratosis congenita (Armanios, Chen et al. 2007; Blasco 2007).

Table 1.1 Telomere function (Rocco, Costagliola et al. 2001; Zou, Yi et al. 2002; Teixeira and Gilson 2005)
• Prevent chromosome ends from being recognized as double-stand breaks. • Regulate cellular proliferation (Replicative Aging/tumor prevention) • Regulate cellular survival • Chromosome positioning • Prevent DNA recombination • Role in mitotic division • Role in meiotic division • Telomere Position Effect (TPE) • Participate in karyotype evolution / speciation

3. Evolution of telomeres

3.1. Unicellular organisms

Telomerase-based end maintenance is likely to be a very ancient mechanism since it is found in widely divergent species that represent many of the major eukaryote lineages (ciliates, animals, fungi, green plants). The loss of telomerase is a catastrophic event unless there is immediate (within a few generations) replacement by an alternative system.

In 1978, Elizabeth Blackburn found that the telomeres of the ciliated protozoan *Tetrahymena thermophila,* consisted of a simple sequence of the hexameric repeat of nucleotides TTGGGG (Blackburn and Gall 1978). Telomerase is necessary for the replication of chromosome ends in this protozoan, and telomeric elongation activity

occurs massively during the macronuclear development when telomeres are formed and replicated (Greider and Blackburn 1985). Elongation by recombination is also seen as a backup mechanism in yeast (Lundblad and Blackburn 1993; DeLange 2004). In the protozoan *Oxytricha fallax*, the telomeric sequence is similar to that of *Tetrahymena* but the terminal sequence is very short (36 bp) (Pluta, Kaine et al. 1982). Gene conversion based on strand invasion and copy-choice replication has also been observed in *Tetrahymena* (Walter, Bozorgnia et al. 2001).

Easy laboratory cultivation conditions and powerful genetics have resulted in *Saccharomyces cerevisiae*, *Kluveromyces lactis* and *Schizosaccharomyces pombe* being used as crucial model organisms for telomere biology research. *Saccharomyces cerevisiae* (Sc) and *Schizosaccharomyces pombe* (Sp) are almost as different from each other as either is from vertebrates: their ancestors separated about 420-330 million years ago. The telomeric proteins of *S. pombe* are more similar to the mammalian ones (Teixeira and Gilson 2005). In the yeast *Saccharomyces*, (TG_{1-3}) or $TG_{2-3}(TG_{1-6})$ telomere repeats are observed (Teixeira and Gilson 2005). In other fungi $(TTAGGG)_n$ is observed in *Cladosporium* but more complex repeats such as (ACACCAAGAAGTTAGACATCCGT)n are found in *Candida albicans* (Table 1.2) (Shampay, Szostak et al. 1984; Coleman, McHale et al. 1993; McEachern and Hicks 1993; Sinclair, Richmond et al. 2007). Today's yeast telomerase enzymatic activity appears to be adapted for both TTAGGG and TG-degenerated sequences (Forstemann, Zaug et al. 2003). Telomeres of *Candida parapsilosis* are composed of long tandem

repeats and also t-circle intermediates (Tomaska, McEachern et al. 2004; Nosek, Rycovska et al. 2005). The widespread occurrence of t-circles across eukaryote lineages suggests that t-circles (which permit telomere elongation by rolling-circles replication) may not only represent a backup if telomerase dysfunction occurs, but also may be the ancestral system for telomere maintenance (Fajkus, Sykorova et al. 2005). Telomeres also play an important role in the nuclear architecture in some organisms. In yeast, telomeres are anchored to nuclear membranes through a protein complex (Galy, Olivo-Marin et al. 2000).

In the causative agent of malaria, the intracellular protozoa *Plasmodium falciparum*, telomeres are followed by a non-coding sub-telomere region (TAS), and telomerase not only maintains telomeres, but also participates in the repair of broken chromosome ends. One of *P. falciparum's* telomere associated proteins, a homologue of the yeast Sir2, is required for the establishment of a heterochromatic structure at the telomeres, leading to silencing of sub-telomeric genes. PfSir2 associates with promoter regions of silenced genes involved in antigenic variation (Figueiredo and Scherf 2005). In kinetoplastid pathogens such as *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major* subtelomeres are closely related to antigenic variation, a process which allows the clonal switch of surface antigens, enabling escape from acquired immune responses (Horn and Barry 2005). T-loops have been found in *Oxytricha fallax* and *Trypanosoma brucei*. Although trypanosome telomeres have the same size as human

telomeres, their t-loops are very small (less than 1 kb in length) (Munoz-Jordan, Cross et al. 2001; DeLange 2004).

Other ways exist to overcome terminal telomere loss and are exhibited by viruses, prokaryotes and some eukaryotes. Poxvirus has a covalently-closed hairpin at each end of its dsDNA genome. Controlled nicking of the hairpin provides the 3′OH group that is necessary for DNA replication. The linear DNA of the spirochete *Borrelia burgdorferi* displays a similar strategy. A complication of this replication strategy is the generation of circular dimers requiring a specialized conversion into monomers (DeLange 2004). Retroviruses reverse transcriptase executes a complex terminal jump in order to maintain their chromosome ends and in adenoviruses the solution to the end-replication problem is provided by a terminal protein primer, which is covalently attached to the 5′ ends of its genome (de Jong, van der Vliet et al. 2003; DeLange 2004)

3.2. Plants

In most plants the telomeric sequence $(TTTAGGG)_n$ is observed (Table 2) (Cox, Bennett et al. 1993; Fuchs, Brandes et al. 1995). Both needle and root samples of long-lived trees such as the coastal redwood (*Sequoia sempervirens*) and the bristlecone pine (*Pinus aristata*) (2000 to 5000 year lifespan) were found to have higher average telomere lengths of the longest, mean, and shortest telomeres compared with aged matched medium-lived and short lived trees such as the longleaf pine (*Pinus palustris*) (100-200 years lifespan) (Flanary and Kletetschka 2005). In needle, root, and core samples, long-

lived trees also display higher telomerase activity compared with both short and medium-lived trees. A direct correlation has been found between telomere length and telomerase activity and the expected lifespan of these trees. In the longest lived tree, the Great Basin bristlecone pine (*P. longaeva)* there was no evidence of overall telomere shortening or decrease in telomerase activity with age (up to 3500 years). One living bristlecone tree "Methuselah" had estimated germination at 2838 BC (Schulman 1958; Flanary and Kletetschka 2005; Flanary and Kletetschka 2006).

In almost all angiosperms, telomeric DNA is composed of many repeats of the heptanucleotide TTTAGGG(McKnight, Riha et al. 2002). However, *Alliacaeae*, a group of monocots that includes the onions and *Aloe* seems to be an exception, and several alternative telomeric DNA structures have been proposed (Pich and Schubert 1998). Thus in Asparagales (includes *Allium* and *Aloe*) there have been at least two switch-points in the evolution of telomeres. The first occurred with the replacement of the Arabidopsis-type telomere for a "TTAGGG vertebrate-like" sequence. A low fidelity of telomerase (with implications for telomere-binding proteins) may have favored a second switch point in the ancestor to *Allium*, leading to a still unclear mechanism (Fajkus, Sykorova et al. 2005). It has been proposed that elongation of minisatellite repeats using recombination/replication processes initially compensated for the loss of telomerase function. In more established ALT groups, subtelomeric satellite repeats may replace the telomeric minisatellite repeat while keeping the recombination/replication mechanisms

for telomere elongation in place. Retrotransposition-based mechanisms may also subsequently become established (Fajkus, Sykorova et al. 2005).

Telomeric length is variable among species, from very short telomeres in the plant model Arabidopsis (*Arabidopsis thaliana*) (2–4 kb) to the extremely long telomeres of tobacco (*Nicotiana tabacum*) (up to 150 kb) (Richards and Ausubel 1888; Fajkus, Kovarik et al. 1995). Telomere length also varies within the same species (McKnight, Riha et al. 2002). Despite having much shorter telomeres than mice, telomerase null Arabidopsis generated through a T-DNA disruption of the single *At-TERT* gene can survive up to ten generations (Fitzgerald, Riha et al. 1999; Riha, McKnight et al. 2001; McKnight, Riha et al. 2002). The last five generations of telomerase deficient mutant plants display increased cytogenetic damage and in late-generation chromosome fusions occur in over 40% of the cells, with some cells surviving with only half of their chromosomes. Amazingly, some plants manage to flower and set seeds until the ninth generation (McKnight, Riha et al. 2002). Differences in the consequences of the massive genome damage probably reflect the greater developmental and genomic plasticity of plants. It is known, for example, that chromosomal rearrangements and ploidy changes are better tolerated in plants (Walbot 1996; Fitzgerald, Riha et al. 1999). Telomere dysfunction in plants, leading to end-to-end chromosome fusions, can have a profound effect on chromosome evolution and even speciation (Fajkus, Sykorova et al. 2005). T loops have been found in plants. Extremely large t-loops, up to 50 kb in size, are seen in peas (*Pisum sativum*) (Cesare, Quinney et al. 2003; deLange 2005).

In plants, telomerase is expressed abundantly in reproductive organs and dividing tissues such as the dedifferentiated callus cells but it is expressed at low or undetectable levels in most post-mitotic vegetative organs (McKnight, Riha et al. 2002). Most cell division takes place in the apical meristem, a group of stem cells that gives rise to all tissues including germ-line cells. It is believe these cells and can undergo approximately 1000 divisions from seed to seed and differentiate into an array of cell types that make a shoot, root, and flower (Fajkus, Kovarik et al. 1995; Oguchi, Liu et al. 1999). Therefore we can conclude that it is unlikely that plants use telomere shortening as a tumor protection mechanism (Oguchi, Liu et al. 1999; Forsyth, Wright et al. 2002).

3.3. Metazoa

3.3.1 Invertebrates

3.3.1.1 Lower Metazoan

As an evolutionary bridge between fungi and higher animals, there are the Lower Metazoan includes the phyla Porifera (sponges), Placozoa (*Trichoplax adhaerens*), Cnidaria (corals and jellyfish) and Ctenophora (comb jellies) and are considered an evolutionary bridge between fungi and higher animals (Sinclair, Richmond et al. 2007) (Fig. 1.4). All these phyla display the "vertebrate" telomeric motif, also found in the unicellular metazoan sister group Choanozoa (Traut, Szczepanowski et al. 2007).

The lowest metazoan phylum is Porifera (Fig. 1.4.a) in which many species are reported to present negligible senescence (Finch 1990). Sponge species usually show

continuous growth, long lifespans, and a highly flexible cell lineage determination (Koziol, Borojevic et al. 1998). Species from this phyla are known for their extensive regenerative capacity and use of both sexual and vegetative forms of reproduction (Finch 1990). *In vivo* and *in vitro* studies in marine demosponges *Suberites domuncula* and *Geodia cydonium* exhibit telomerase activity in their somatic and immortal germ tissues. After dissociation into single cell suspensions, isolated cells retain their proliferative capacity but lose telomerase activity, possibly due to lack of contact/adhesion factors. However, telomerase activity is recovered after aggregation of the cells to form primmorphs (Koziol, Borojevic et al. 1998).

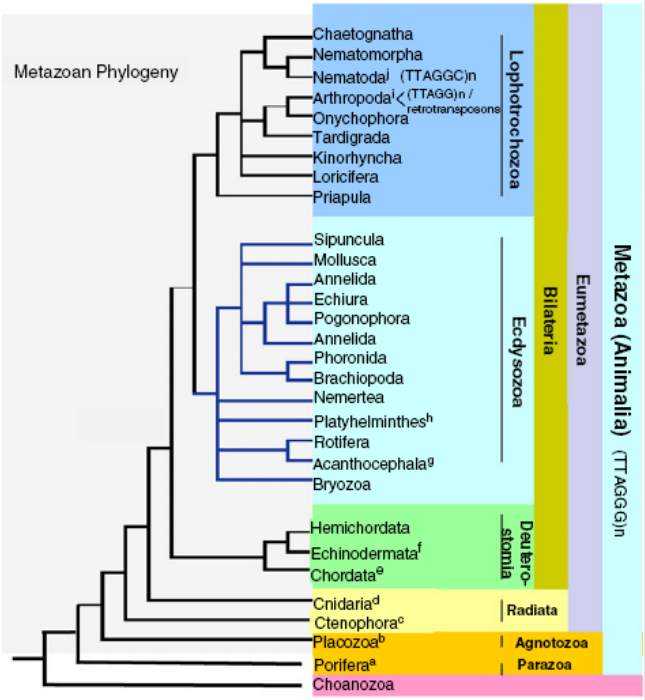
These simple multicellular animals provide excellent models for the study of the separation of soma and germ-cell lineages. In the sponges studied, the number of germ-cells is much reduced or null, so the levels of telomerase observed should come from elevated levels of telomerase in the somatic cells that display unlimited replication potency. Alternatively, there might be a high number of somatic stem cells capable of unlimited replication that would undergo subsequent differentiation. Although Archaeocytes in sponges are pluripotent (stem-cell like), with the potential for differentiation into all major cell types, morphological data seem to support the hypothesis that the proliferation of all major somatic cells types is the major contributor for tissue growth. Furthermore, the plasticity of sex determination and the ability of fully differentiated cells to produce gametes also favor the first hypothesis (Koziol, Borojevic et al. 1998; Muller and Muller 2003).

In Calcarea (*Leucosolenia* sp and *Sycon* sp.) (Fig. 1.4.a) telomere sizes seem to range from below 1 kb to over 20 kb. One study in Calcarea that also examined the demosponge *Suberites* failed to detect telomerase activity in either species (Traut, Szczepanowski et al. 2007). This is unexpected and conflicts with the *Suberites* study cited above, so it is premature to conclude that Calcarea do not express telomerase.

Among Cnidarians (Fig. 1.4.d), the Anthozoans (Corals) are the most basal organism reported to exhibit the $(TTAGGG)_n$ telomeric sequence. This repeat is found in DNA from several Scleractian order corals: *Acropora surculosa*, *Leptoria phrygia*, *Favia pallida* and *Goniastrea retiformis*. Average telomere length of *Acropora surculosa* is 3.5 kb (Sinclair, Richmond et al. 2007). Reef corals display vegetative growth of hundreds of years, their rate of mortality decreases as coral body mass increases and several species tend to behave as plants, increasing fecundity as the colonies grow larger (Finch 1990). In spite of these properties, which are characteristic of negligible senescence, reef corals show signs of aging, with declining growth, calcification and reproduction before colony death in *Stylophora pistillata* (Rinkevich and Loya 1986; Finch 1990).

Cnidaria Scyphozoa species (Fig. 1.4.d) such as compass jellyfish (*Chrysaora hysoscella*) and blue jellyfish (*Cyanea lamarckii*) and Ctenophora (*Pleurobrachia pileus*) (Fig. 1.4.c) reported telomere sizes range from less than 1 kb to over 20 kb. In the Cnidaria *Hydra vulgaris* (Fig. 1.4.d) sizes seem to be around 20 kb. Telomerase activity has been found in gonad extracts of Cnidaria moon jelly (*Aurelia aurita*) and the ctenophore (*Pleurobrachia pileus*). However, similar studies in Cnidarians such as hydra

or in Placozoan (*Trichoplax*) (Fig. 1.4.b) did not detect telomerase activity (Traut, Szczepanowski et al. 2007).



	Phylum	Classes	Orders
a.	Porifera	Calcarea Demospongia	
b.	Placozoa	Tricoplacia	
c.	Ctenophora	Tentaculata	
d.	Cnidaria	Anthozoa Scyphozoa Hydrozoa	Scleratinia
e.	Chordata	Subphyla: Cephalochordata Urochordata (class Ascidiacea) Vertebrata	
f.	Echinodermata	Echinoidea	
g.	Acanthocephala	Palaeacanthocephala	
h.	Platyhelminthes	Trematoda Monogenea Cestoda	
i.	Arthropoda	Insecta	Coleoptera Lepidoptera Diptera Odonata
		Malacostraca	Decapoda
		Pycnogonida	
		Arachnida	Araneae
j.	Nematoda	Secernentea	

Fig. 1.4- Phylogenetic tree of metazoa (animalia). The tree and chart shows the relationships of the different species whose telomere biology is discussed, keyed to superscript letters.

3.3.1.2 Bilateria invertebrates

Among Bilateria (Fig. 1.4), the phyla Onychophora, Platyhelminthes, most Annelida and Mollusca, Echinodermata and the subphylum Urochordata (Fig. 1.4.e),

seem to share the "vertebrate" telomere motif $(TTAGGG)_n$ (Jha, Dominguez et al. 1995; Joffe, Solovei et al. 1998; Vitturi, Colomba et al. 2000; Wang and Guo 2001; Castro and Holand 2002; Plohl, Prats et al. 2002; Laird and Weissman 2004; Vitkova, Kral et al. 2005).

3.3.1.3 Ecdysozoa (Platyhelmintes and Acanthocephala)

In the Trematode *Schistosoma mansoni* (Fig. 1.4.h) chromosomes are also protected from degradation by telomeres (Hirai and LoVerde 1996). A telomeric study of parasitic worms including the Platyhelminthes flatworm groups Monogenea and Cestoda, and thorny-headed worms (Syndermata: Acanthocephala) revealed conservation of the (TTAGGG)n sequence, in Monogenea (*Paradiplozoon homoion*) and Cestoda (*Caryophyllaeus laticeps*, *Caryophyllaeides fennica*, and *Nippotaenia mogurndae*. However neither this motif or the nematode motif were present in the parasitic Acanthocephala (*Pomphorhynchus laevis* and *Pomphorhynchus tereticollis*) (Fig. 1.4.g) suggesting the existence of an as yet unknown telomeric repeat sequence or an alternative mechanism of telomere maintenance (Bombarová, Vítková et al. 2009).

3.3.1.4 Lophotrochoa (Nematodes and Arthropods)

The so called nematode motif $(TTAGGC)_n$, is found in the Secernentea roundworms *Ascaris lumbricoides*, *Ascaris sum* and *Parascaris univalens* (Fig. 1.4.j) (Niedermaier and Moritz 2000). In *Ascaris*, chromatin fragmentation involves a complex molecular mechanism that includes site-specific chromosome breaks, telomeric synthesis,

and degradation of DNA (Muller, Wicky et al. 1991; Traut, Szczepanowski et al. 2007). In *Parascaris univalens* the haploid germline genome is contained in a single large chromosome and the somatic genome is surrounded by heterochromatin (HET) blocks constituted by segments of the repeats TTGCA and TTTGTGCGTG. However, in both species, the ends of the germline chromosomes are said to be capped by the same $(TTAGGC)_n$ tracts, which are added to all the new somatic ends after removal of the old ones during the complex chromatin diminution process (Niedermaier and Moritz 2000). Chromosome capping in the free-living nematode *Caenorhabditis elegans*, is achieved by the 4-9 kb telomeric repeats $(TTAGGC)_n$ (Wicky, Villeneuve et al. 1996).

All the major arthropod Subphyla (Chelicerata--except spiders, Myriapoda, Crustacea and most Hexapoda) (Fig. 1.4.i) have the $(TTAGG)_n$ telomere motif (Traut, Szczepanowski et al. 2007). Unlike mammals that stop growing after adulthood, some invertebrates, such as the Decapoda crustacean lobster (*Homarus americanus*) grow continuously throughout life, although growth rates seem to decrease with age. Lobsters show asymptotic growth and can occasionally weigh over forty pounds, and seem to present negligible or very slow gradual senescence. Lobsters have very long lifespans of 50 to 100 year and neither sex exhibits a post-reproductive phase nor molting cessation. They are also able to regenerate their limbs even at advanced ages (Finch 1990; Klapper, Kuhne et al. 1998). Telomere analysis reveals the sequence $(TTAGG)_n$ and telomerase expression has been found in fully differentiated tissues of all organs, with high levels detected in the hepatopancreas and heart and moderate levels in skin and muscle tissues (Klapper, Kuhne et al. 1998). Tumors are rare in adult lobsters and do not seem to

correlate with size or lifespan (Finch 1990). Another Decapoda crustacean, the green sea crab (*Carcinus maenas*) also has the pentameric $(TTAGG)_n$ telomere sequence and high telomerase activity in its tissues (Elmore, Norris et al. 2008).

The low number of tumor reports in decapod crustaceans may represent a truly low incidence of neoplasia compared to other well studied animal groups rather than insufficient information. This is a large animal group of more than 10,000 species, many commercially important and well investigated, such as lobsters, crabs, shrimp and crayfish. Despite many of these species having long lifespans, some reaching almost 100 years, neoplasias are said to be extremely rare (Vogt 2008). Furthermore, many of these species are benthic, and have an elevated exposure to carcinogens but the frequencies of tumors are remarkably different from mollusks, bottom feeding fish and other fish and even insects (Vogt 2008).

The reason for the low cancer incidences observed in this Phyla are unknown, but many mechanisms may play a role in this event. Decapod crustaceans exhibit some remarkable carcinogen detoxification pathways such as rapid elimination of PAH-related DNA adducts from the tissues. Their immune system includes only innate responses and is reported to be able to either phagocytose or melanize and encapsulate all kinds of foreign material. Arthropods use this rigid melanin barrier to isolate and, together with quinolone cellular toxicity, eliminate cancer cells and damaged tissue areas (Vogt 2008). Stem cell maintenance until the end of life, for example by telomere protection due to high telomerase activity in tissues throughout life, has also been suggested as contributors

for the virtual absence of age-related cancer in the Decapoda (Vogt 2008). These species may provide excellent models for tumor protection mechanism studies.

With the exception of the heterogeneous Coleoptera, most insect orders can be divided into those that use the telomeric repeat $(TTAGG)_n$ (e.g. Lepidoptera) or the ones that do not (e.g. Diptera) (Okazaki, Tsuchida et al. 1993; Meyne and Imai 1995; Sahara, Marec et al. 1999; Frydrychova, Grossmann et al. 2004; Sinclair, Richmond et al. 2007).

Telomerase activity has recently been detected in crickets, cockroaches, and species of Lepidoptera (Sasaki and Fujiwara 2000). The telomerase reverse transcriptase (TERT) subunit has been identified and characterized in the domestic silkworm (*Bombyx mori*) and the flour beetle (*Tribolium castaneum*) (Osanai, Kojima et al. 2006). In the group of insects with the largest number of species, the beetle (order Coleoptera), the telomerase-dependent (TTAGG)*n* motif has been repeatedly lost (5 to 6 times) in different phylogenetic branches and was likely replaced with the alternative mechanisms of telomere elongation (Frydrychova and Marec 2002). The order Diptera seems to be an exception from the general pattern of having short G-rich repeats at their telomeres, and instead often has arrays of complex long satellite repeats at the ends of their chromosomes (e.g. *Chironomus* & *Anopheles gambiae*) (Rosen and Edstrom 2000; Walter, Bozorgnia et al. 2001; Traut, Szczepanowski et al. 2007). Elongation of telomeres in the mosquito (*Anopheles*) is done through gene conversion between complex terminal satellite repeats that are present at natural telomeres (Walter, Bozorgnia et al. 2001). One hypothesis is that Diptera may have lost the telomerase gene and was forced

to use alternative mechanisms of telomere elongation (Biessmann and Mason 1997; Walter, Bozorgnia et al. 2001). The fruit fly (*Drosophila melanogaster*) uses telomerase independent mechanisms such as chromosome end capping with non-LTR retrotransposons. Chromosome end-elongation is predominantly achieved by terminal insertion of two classes of telomere-specific LINE-like retrotransposable elements, HeT-A and TART (Mason and Biessmann 1995). However, *Drosophila* telomeres can also be extended by gene conversion (Mikhailovsky, Belenkaya et al. 1999) and perhaps by recombination between telomeric HeT-A elements (Kahn, Savitsky et al. 2000). The telomeric structure of Damselflies (Zygoptera) and spiders (Araneae) is still unclear (Frydrychova, Grossmann et al. 2004; Vitkova, Kral et al. 2005). Sea spiders (Pycnogonida) also have the (TTAGG)n telomeric motif (Traut, Szczepanowski et al. 2007).

3.3.1.5 Deuterostomia

In Deuterostomia, which includes the phyla Chordata (Fig. 1.4.e and 1.5.a) and Echinodermata (e.g. sea urchins) (Fig. 1.4.f), many examples of long-lived species have been found. Longevities of a decade or more are found in many sea urchins, and in fact, mortality rates decrease with size in adults (Finch 1990). The Red Sea urchin (*Strongylocentrotus franciscanus*) (Fig. 1.4.f) grows indeterminately during a lifespan that can go beyond 100 years without evidence of age-related disease or decline in reproductive potential, while other species such as the green sea urchin (*Lytechinus variegatus*) are fast growing and short lived, with a maximum lifespan of 3 to 4 years.

Telomere studies in the Red Sea urchin reveals telomerase activity in mature eggs, and also during early stages of development of *L. variegatus* and in tissues during adulthood in both species (Aristotle's lantern muscle, ampullae, esophagus, intestine, tube feet, male and female gonads). The $(TTAGGG)_n$ telomeric sequence has been found in the moderately long-lived species *S. purpuratus.* Long telomere lengths (>20 kb) were found both in germ and somatic tissues of *L. variegatus*. The adult tissues of *S. franciscanus* have short telomere lengths (≈ 5 kb), similar to the California purple sea urchin (*S. purpuratus*) (6 kb), and no telomere shortening occurs throughout life of these species (Lejnine, Makarov et al. 1995; Francis, Gregg et al. 2006). It is also known that sea urchin embryo telomeres need to be maintained. The use of cationic porphyrins as telomere interfering agent decreases the rate of cell proliferation and leads to increased chromosome destabilization (Izbicka, Nishioka et al. 1999). These results seem to indicate that neither short nor long-lived sea urchins use replicative aging as a tumor protective mechanism (Francis, Gregg et al. 2006). Furthermore, the number of reported cases of neoplasia in sea urchins, a very intensively studied model organism, is very low (www.pathology-registry.org). This suggests that these species have evolved other mechanisms of tumor prevention/suppression, such as efficient cellular or molecular protection against damage or free radicals and/or a good capacity of replenishment to damaged cells (Francis, Gregg et al. 2006). These species may be excellent candidates for future senescence and tumor protection mechanism studies (Francis, Gregg et al. 2006).

The golden star tunicate (*Botryllus schlosseri*), the model Urochordate (Fig. 1.4.e and Fig. 1.5.b), is a colonial organism that propagates both asexually and sexually during the 2 to 5 years of colony life. Asexual budding occurs continually from the progenitor body wall and when the colony reaches a critical size sexual reproduction initiates with the production of gonads. It has been proposed that pools of stem cells assure renovation throughout the lifespan. Heterogeneous telomeres of 6-15 kb protect the chromosome ends and high levels of telomerase have been reported in germ and embryonic tissues (Laird and Weissman 2004). Telomerase activity peaks in tissues containing bud rudiments, then decreases in buds that are going through organogenesis and drops to even lower levels in functional zooids, in individual organs and blood (Laird and Weissman 2004). It has been hypothesized that telomerase activity needs to be retained in progenitor and stem cells, is downregulated during differentiation, and is not necessary to maintain the relatively short-lived somatic tissues of *Botryllus* (Laird and Weissman 2004).

Information about telomere sequences and telomerase TERT and TR/TERC sequences and structure in invertebrates and vertebrates is now readily available online (Table 1.2) (Podlevsky, Bley et al. 2007).

Table 1.2. Telomere sequences and replicative aging during evolution (Niedermaier and Moritz 2000; Podlevsky, Bley et al. 2007; Traut, Szczepanowski et al. 2007).

Group /Specie		Telomere sequences	Telomere-based replicative aging
Vertebrates	Mammals	TTAGGG	Probable in many Orders
	Birds		Probable in many Orders
	Reptiles		Not likely
	Amphibians		No
	Fish		
Invertebrates (Urochordata)	Sea Squirts (*Ciona intestinalis* and *Ciona savignyi*)		
Echinodermata	Purple Sea Urchin (*Strongylocentrotus purpuratus*)		
Invertebrates (Mollusca)	Wedgeshell Clam (*Donax trunculus*)		
	Bay scallop (*Argopecten irradians*)		
Invertebrates (Porifera)	Sponges		
Invertebrates (Cnidaria)	Corals and jellyfish		
Invertebrates (Ctenophora)	comb jellies		
Invertebrates (Placozoa)	*Trichoplax adhaerens*		
Invertebrates	Choanozoa		
Invertebrates	Freshwater shrimp (*Gammarus pulex*)	TTAGG	
	Lobster (*Homarus americanus*)		
Invertebrates (Insects)	Insects (except some coleoptera and Diptera)		
	Fruit Fly (*Drosophila melanogaster*)	Retrotransposons	
	Fly (*Drosophila virilis*)	Retrotransposons Satellite sequence	
	Fly (*Chironomus tentans*)	Satellite sequence	
	African malaria mosquito (*Anopheles gambiae*)	Unequal recombination/ gene convertion	
Invertebrates (Nematodes)	*Ascaris lumbricoides, A. sum*	TTAGGC	
	Parascaris univalens	TTAGGC	
Fungi (Saccharomycotina)	Fission yest (*Schizosaccharomyces pombe*)	$G_{2\text{-}8}$TTAC(A)	
	Baker's yeast (*Saccharomyces cerevisiae*)	$T(G)_{2\text{-}3}(TG)_{1\text{-}6}$	
	Candida albicans	ACGGATGTCTAACTTCTTGGTGT	
	Others	Diverse complex sequences	
Fungi (Pezizomycotina)	Most	TTAGGG	
	Aspergillus oryzae	TTAGGGTCAACA	
Fungi (Basidomycotina)	*Cryptococcus neoformans*	$TTA(G)_{4\text{-}6}$	
Mold	*Dictyostelium discoideum*	$A(G)_{1\text{-}8}$	
	Physarum polycephalum	TTAGGG	
	Didymium iridis		
Plants	*Plants sp*	TTTAGGG	
Plants (Eudicots)	Common Tabacco(*Nicotiana tabacum*)	TTAGGG	
	Tomato (*Solanum lycopersicum*)	TT[T/A]GGG	
	Italian olive ash (*Strombosia pustulata*)	TTTTAGGG	
Plants	*Aloe* sp.	TTAGGG	
	Hyacinthella dalmatica		
	Othocallis siberica		

Algae	Green Alga (*Chlamydomonas reinhardtii*)	TTTTAGGG	
Ciliates (Oligohymenophorea)	*Tetrahymena thermophila*	TTGGGG	
	Paramecium sp.	TT[T/G]GGG	
Ciliates (Spirotrich)	*Euplotes* sp.	TTTTGGGG	
	Oxytricha sp.	TTTTGGGG	
Other Protists	*Plasmodium* sp.	TT[T/C]AGGG	
	Theileria annulata	TTTTAGGG	
	Cryptosporidium parvum	TTTAGG	
	Giardia lamblia	TTAGG	
	Giardia intestinalis	TAGGG	
	Leishmania major	TTAGGG	
	Trypanosoma brucei	TTAGGG	

3.3.2 Vertebrates (Fig. 1.4.e and 1.5.c)

The telomere sequence $(TTAGGG)_n$ is conserved in the phylum Chordata and is thought to have arisen 400 million years ago (Meyne, Ratliff et al. 1989). The essential core structure of telomerase RNA seems to be preserved in vertebrates (Chen, Blasco et al. 2000).

3.3.2.1 Fish

Several fish species can grow throughout life with high proliferative capacity displayed by all somatic cells (Patbaik, Mahapatro et al. 1994). In many other species, organs continue to grow and growth after the larval stage is dependent on both cellular hyperplasia and hypertrophy (Mommsen 2001; Lau, Wong et al. 2008).

Among Elasmobranchs (Fig. 1.5.d), the dogfish shark (*Squalus acanthias*) is the longest lived (70 years) but the reported lifespan of most cartilagenous fish is much lower than 15 years in captured specimens (Finch 1990). Telomeric $(TTAGGG)_n$ sequences are present in cartilagenous fish (Rocco, Costagliola et al. 2001). Dogfish shark (*Squalus*

acanthis) has human-like telomeres (10-15 kb) and high levels of telomerase expression (McChesney, Elmore et al. 2004/2005; Elmore, Norris et al. 2008). Telomere bands of 3 kb seem to be common to four species of Batoidea (*Torpedo marmorata*, *Torpedo ocellata*, *Raja asterias*, *Raja montagui*) and two species of Galeomorphii (*Mustelus asterias*, *Scyliorhinus stellaris*). In the little skate (*Raja erinacea*) telomeres ranged between 10-15 kb and in other rays, intense short telomeric bands varying in length from 0.5 to 2 kb, were observed (Rocco, Costagliola et al. 2001; Elmore, Norris et al. 2008). Telomeric sequences in the paracentromeric and/or interstitial regions was observed in chromosomes of two Batoidea, the blue-spotted stingray (*Taeniura lymma*) and the electric ray (*Torpedo ocellata*). This finding supports the hypothesis that in cartilaginous fish Robertsonian fusions involving telomeres could have led to an increase in bi-armed chromosomes and a decrease of the acrocentric ones, thus playing an important role in karyotype evolution (Rocco, Costagliola et al. 2001; Rocco, Morescalchi et al. 2002).

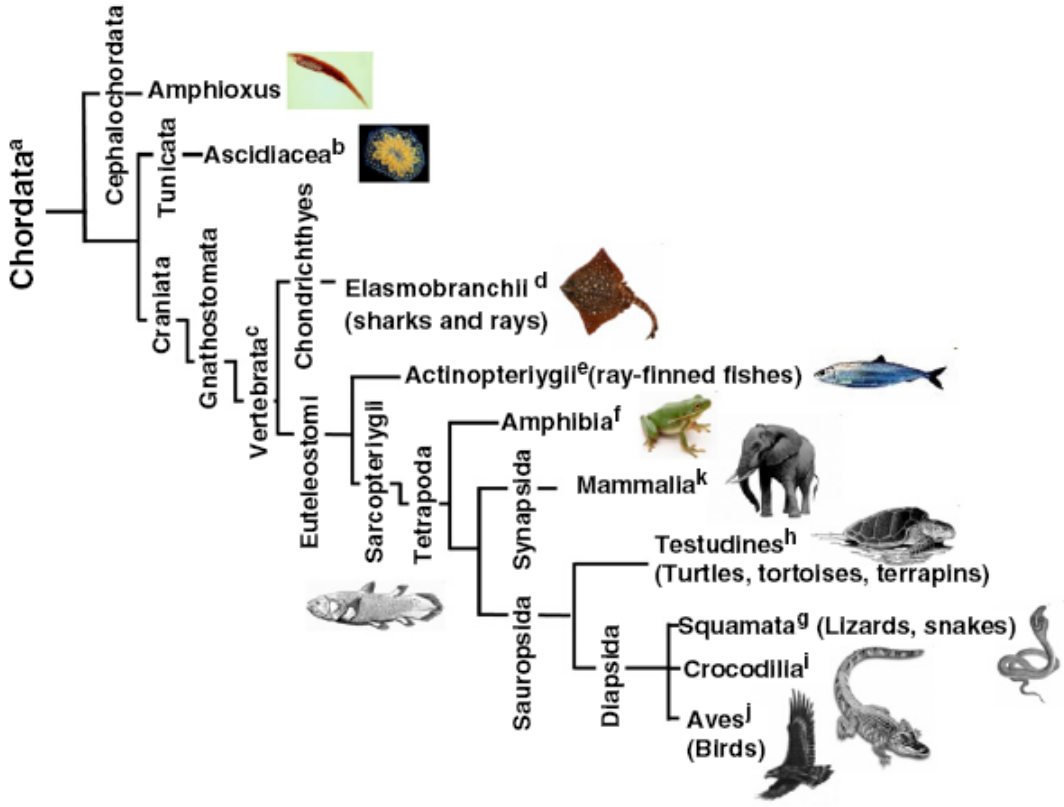


Figure 1.5. Phylogenetic tree of the phylum chordate. The tree shows the relationships of the different chordates whose telomere biology is discussed, keyed to superscript letters.

Teleosts (bony fish, within the class Actinoptergii) (Fig. 1.5.e) represent more than half of the forty to fifty thousand vertebrate species (Finch 1990). Many reports show that eels, sturgeons, and other teleosts can live 80 years or more. In teleosts the record lifespan of 152 years is held by the lake sturgeon (*Acipenser fulvescens*) and the beluga sturgeon (*Huso*) (118 years), reaching weights of over 3 tons (Tsepkin and Sokolov 1971; Finch 1990). Teleost fishes exhibit different patterns of aging. The pacific salmon (*Oncorhynchus*) and eel (*Anguilla anguilla*) exhibit rapid senescence and death at

first spawning, while other fish such as medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) seem to display gradual "mammalian-like" senescence (Kishi, Uchiyama et al. 2003). In Cyprinidae, species with very different lifespans such as carp (*Cyprinus carpio*, which may live more than 100 years) and zebrafish (*Danio rerio*), which has a lifespan of approximately 5 years) exhibit growth characteristics that imply very slow senescence (Kishi, Uchiyama et al. 2003). The short life, short generation time (3-5 months), and seemingly unlimited capacity to regenerate their fins in 7-10 days of zebrafish place it in a privileged spot as a genetically tractable vertebrate model for studying functional aging, where genetic mutant screens could be used to study gradual senescence (Johnson and Bennet 1999; Kishi, Uchiyama et al. 2003). Small telomeric sizes have been reported in teleosts: zebrafish (*Dana rerio*) (2-10 kb); killifish (*Fundulus heteroclitus*) (2-10 kb), japanese medaka (*Oryzias latipes*) (3-12 kb) and american eel (*Anguilla rostrata*) (10-15 kb) (Elmore, Norris et al. 2008). In trout (*Oncorhynchus mykiss*), erythrocytes have larger average telomeric terminal restriction fragment (TRF) lengths of 20 kb (Lejnine, Makarov et al. 1995).

Telomerase activity is detected in cells and tissues of several teleost fish (e.g. fugu, zebrafish, rainbow trout, Japanese medaka, flounder) (Bradford, Miller et al. 1997; Klapper, Heidorn et al. 1998; Kishi, Uchiyama et al. 2003; McChesney, Elmore et al. 2004/2005). The integral telomerase RNA (TR/TERC)) from five teleost fish, *Danio rerio, Oryzias latipes, Gasterosteus aculeatus, Takifugu rubripes* and *Tetraodon nigroviridis* has been characterized (Xie, Mosig et al. 2008). The gene encoding the TERT subunit of telomerase has been isolated and cloned in pufferfish (*Fugu rubripes*)

and zebrafish (Yap, Yeoh et al. 2005; Lau, Wong et al. 2008). In *Fugo*, the fTERT mRNA is found at low levels in several tissues such as skin, stomach, spleen, heart, brain, and eye, with high expression in the gill, testis and ovary. fTERT expression is detected in an immortalized eye-derived cell line from *Fugu*. The level of expression is higher in actively dividing cells and is reduced at quiescence, suggesting cell cycle regulation of TERT (Yap, Yeoh et al. 2005). In zebrafish, TERT mRNA expression and telomerase activity correlate closely and are detected in all somatic tissues, including retina and brain, with the highest activities found in gills and in the ovary, where the highly proliferative germ cells are found (Lau, Wong et al. 2008). Telomerase activity is found in several somatic tissues of the American eel (*Anguilla rostrata*)(McChesney, Elmore et al. 2004/2005).

Since significant levels of telomerase have been detected at both short and long lived aquatic species, it's been suggested that the expression of telomerase in fish is likely related to tissue regeneration and not lifespan (Elmore, Norris et al. 2008). Telomere lengths in fish species are short 'human-like' and are maintained by an increase in telomerase activity during regeneration of injured tissues of killifish, Japanese medaka, and zebrafish (Elmore, Norris et al. 2008).

The different patterns of senescence reported in fish make them unique models for studying the aging process. Most marine species with their high regenerative capacities and long lifespans seem to maintain telomerase in their tissues. The lack of telomerase repression in somatic tissues suggests that they do not use telomere shortening and

replicative aging as a tumor-protection mechanism. Many of these species may prove excellent models for studies in regeneration, stem cells, DNA repair, cancer and aging.

3.3.2.2 Amphibians (Fig. 1.5.f)

Senescence and mortality rates in the class Amphibian are not well studied but relatively long lifespans have been reported, mainly among the larger species, such as the giant salamandra (*Megalobachus japonicus*), which can live at least 55 years and the toad (*Bufo*) that can reach at least 36 years (Finch 1990). Many other species exceed the age of 15 years. Increase in fitness with age is reported in some species such as bullfrogs (*Rana catesbeiana*) (Finch 1990). Most data on the experimental model African clawed frog (*Xenopus*), which can live at least 15 years, suggests that senescence in amphibians is negligible or very slow (Finch 1990). *Xenopus* telomeres range from less than 10 kb to over 50 kb, in a polymorphic pattern between individuals (Bassham, Beam et al. 1998). Mud puppy (*Necturus maculosus*) erythrocytes have been found to have huge telomere lengths (average 100kb) (Lejnine, Makarov et al. 1995). Unusual inheritance patterns of some bands are observed when *Xenopus* telomeres from whole embryos are compared to telomeres in parent spleens. In some crossings the telomeres of the embryo or in the male testis are shorter than the telomeres of the parents' spleen, consistent with a significant amount of DNA rearrangement at telomeres. Telomere length regulation of *Xenopus* may be different from that reported in mammals. Telomere data in *Xenopus* is also consistent with the occurrence of some degree of meiotic rearrangement (Bassham, Beam et al. 1998). A TERT gene from *Xenopus*, designated xTERT has been identified (Kuramoto,

Ohsumi et al. 2001). Telomerase activity is found in oocytes, embryos, and tissues from adult frogs (>1-2 years, *Xenopus laevis*). Telomerase activity is most abundant in testis, spleen, liver, and embryos (Mantell and Greider 1994; Bousman, Schneider et al. 2003). In brain and muscle tissues telomerase activity is lower but still readily detectable. Furthermore, this activity does not seem to be limited to the polyploid members of the genus since telomerase activity is also found in somatic tissues of the diploid *Xenopus tropicalis* (Bousman, Schneider et al. 2003).

3.3.2.3 Reptiles (Fig. 1.5.g,h,i)

The sequence $(TTAGGG)_n$ has been documented in species from the Squamata orders Sauria and Serpentia (Fig. 1.5.g) (Meyne, Ratliff et al. 1989). Among adult garter snakes (*Thamnophis elegans*) telomeres range between 16-25 kb and decrease with age (Bronikowski 2008). However, in water pythons (*Liasis fuscus*) telomere length did not change between 1 and 20 years of age. In adult pythons, reported telomere length was about 28 kb. Telomeres of hatchling pythons (about 7 kb) were significantly shorter than from one-year-old adults. It has been hypothesized that since hatchlings show high somatic cellular proliferation rates, the increase in telomeric length may have been caused by increased telomerase activity (Ujvari and Madsen 2009). Some lizards are known to have excellent tissue regeneration capacity. Telomerase activity has been observed in all tissues of the six-lined racerunner (*Cnemidophorus sexlineatus*), a teiid lizard (lifespan 4 years) (Christiansen, Henderson et al. 2001). The same study found that skin fibroblasts of a juvenile blue racer (*Columber constrictor*) can undergo more than

124 population doublings (PD) with strong telomerase activity detected after 100 PD, which is suggestive of immortalization of the culture (Christiansen, Johnson et al. 2001). In another lizard, the Carolina anole (*Anolis carolinensis*), cellular proliferative capacities were greater than human diploid cells (Simpson and Rauch 1989). Telomere length (27–34 Kb) in erythrocytes from the Crocodilia American Alligator (*Alligator mississippiensis*) decreases with body length, and telomere lengths inversely correlate with age in the Chinese Alligator (*Alligator sinensis*) (Fig. 1.5.i) (Scott, Haussmann et al. 2006; Min, Xiao-Bing et al. 2009).

Among Testudines (Fig. 1.5.h), turtles have been reported to live more than 100 years, in captivity and have very high annual survival rates in natural conditions. Senescence has not been proven to occur in these species. Mortality does not seem to increase during aging, the reproductive capacity of females grows during their lifespan, and apart from carapace alteration from soil abrasion, no age-specific diseases are known (Girondot and Garcia 1999). Studies in mature breeding sea turtles (*Chelonia mydas*) have reported an absence of a decline in growth rate (Carr and Goodman 1970).

Population doublings of 100-130 have been observed in fibroblast cultures from young Galapagos tortoises (*Geochelene nigra*) (Goldstein 1974). Another turtle species (*Pseudemys scripta*) has been found to have long average telomere lengths (≈ 50 kb) (Lejnine, Makarov et al. 1995). However, in a study, cell culture senescence has been observed between PD 18-45 in yellow mud turtles (*Kinosternon flavescens*), which have a lifespan of 30-45 years. It has been shown that fibroblasts from hatchlings undergo

about twice as many population doublings in culture as those from 25 year old mud turtles. Telomere shortening of about 30-50% was observed between hatchlings and adults, and apart from the gonad, no telomerase was found in tissues from these turtles (Christiansen, Johnson et al. 2001). More studies are needed to clarify if this cellular growth arrest is due to culture stress from inadequate growth conditions leading to stasis or from telomere-based replicative aging. In the same study, cells from the long-lived snapping turtle (*Chelydra serpentina*), (lifespan over 100 years) reportedly multiplied in culture for over 265 PD. In these snapping turtles, telomerase activity went from barely detectable at 157 PD to very strong at 191 PD. Telomerase activity was also detected in old painted turtles (*Chrysemys picta*) and cultured cells from this species were still dividing well at PD 120. Painted turtles present continuous growth during their lifespan of over 60 years and don't show reproductive senescence with age. In this species, telomeres don't seem to vary with age and range above 60 Kb (Paitz RT, Haussmann MF et al. 2004). Telomerase activity was found in gonads of two ornate box turtles (*Terrapene ornata*) hatchlings and in other organs of one of them (Christiansen, Johnson et al. 2001). Telomerase activity has therefore been found in two divergent families of turtle (Chelydridae and Emydidae).

Telomeres of about 20 kb were found in both embryos and adult erythrocytes in European freshwater turtles (*Emys orbicularis*). This species has a similar longevity to humans but is not known to display signs of senescence. Telomeric shortening did not occur in European freshwater turtles, but information about telomerase activity in the tissues of this species and many other species of Chelonian (and Reptilia in general) is

not available (Girondot and Garcia 1999). The available data suggests that telomerase is often found in adult somatic tissues of reptiles and telomere based replicative senescence is unlikely to occur in most of the species studied to date.

3.3.2.4 Birds (Fig. 1.5.j)

Birds (class Aves) and other homeothermic vertebrates exhibit gradual senescence with a definite lifespan (Finch 1990). Also, bird species are clearly longer lived than mammals of similar body weight (up to 3 times longer) (Holmes, Fluckiger et al. 2001). This finding of slow senescence rates is a paradox since, compared to similar sized-mammals, birds have 2-2.5 times higher metabolic rates, higher body temperatures (3^{o}C higher) and elevated glucose levels (two-to four-fold). According to most biochemical theories of aging this should have led to increased tissue cellular damage and accelerated aging (Holmes, Fluckiger et al. 2001).

In Aves, rates and patterns of aging can be extremely variable among different orders. In Galliformes, including the domestic chicken (*Gallus gallus*) and quail (*Coturnix coturnix*) short lifespans and fast aging rates have been observed. Exceptionally long-lived for their body size are some raptors (Falconiformes), hummingbirds (Apodiformes), parrots (Psittaciformes), sea birds (Charadriiformes), and songbirds (Passeriformes). Delayed maturity and low annual fecundity have been linked to slow avian senescence (Holmes, Fluckiger et al. 2001). Some birds, such as the male zebrafinch (*Taeniopygia guttata*) have shown neuroregeneration capacity during song

learning (Scharff, Kirn et al. 2000). Prevention of tissue damage by ROS or glycosylation endproducts has also been reported (Holmes, Fluckiger et al. 2001).

In Galliformes, chicken (*Gallus gallus*) telomeric DNA represents at least 3 to 4% of the genomic DNA, about 10 times higher than what has been found in the human genome. Three overlapping sizes of telomere arrays are found in Southern blot analysis of chickens and classified as: Class I (0.5 to 10 kb), Class II (10 to 40 kb) and Class III (200 kb to 3 Mb) (Delany, Krupkin et al. 2000; Rodrigue KL, May BP et al. 2005). Class I bands do not exhibit age-related telomere shortening and are resistant to digestion by Bal 31 exonuclease, indicating that these arrays are located internally rather than at the end of the chromosomes (Delany, Krupkin et al. 2000; Delany, Daniels et al. 2003). Chicken and primitive Palaeognathae birds commonly exhibit truly interstitial (non-centromeric and non-telomeric) $(TTAGGG)_n$ sites (Nanda, Schrama et al. 2002). Chicken Class II telomeres seem to shorten with age, similarly to human telomeres (Delany, Krupkin et al. 2000). Class III "mega-telomere" arrays are the largest reported in all vertebrates and are located at chromosome ends by Bal 31 exonuclease analysis. These highly polymorphic elements map to the 7 to 23 Mb microchromosomes (Delany, Krupkin et al. 2000). These arrays were mapped to four autosomes and one sex chromosome (one array per chromosome). The female-specific array (2.8 Mb) was mapped to the q arm the female-specific sex chromosome (Rodrigue KL, May BP et al. 2005). Mega-telomere number and distribution is variable but two mega-telomere loci (GGA 9 and GGAW) are common among diverse chicken genetic lines. The same study

reports that the DF-1 cell line contains the greatest amount of telomeric sequence per genome (17%), as compared to UCD 001 (5%) and DT40 (1.2%) (O'Hare and Delany 2009). Studies in inbred chicken lines showed a hyper-variable inheritance pattern suggestive of a high degree of recombination of these Class III arrays (Rodrigue KL, May BP et al. 2005).

The chicken telomerase reverse transcriptase (chTERT) component has been well characterized (Delany and Daniels 2004). Telomerase activity is high in early stage embryos and during organ development but is down-regulated during late embryogenesis or postnatally in most somatic tissues. Renewable tissues such as reproductive and immune organs seem to retain high levels of telomerase activity even in adults (4-5 years). Telomerase activity in chickens tends to correlate with the proliferative potential of the tissue. The telomere arrays of the somatic and germ tissues in the embryo display similar telomeric sizes, but telomeres in adult somatic tissues arrays are shorter, exhibiting an average decrease in size of 3.2 kb. Telomere shortening is detected in telomerase positive adult tissues (kidney, intestine, spleen), a pattern also reported in some human tissues (Hiyama, Kiyama et al. 1996; Delany, Daniels et al. 2003). Primary cultures of embryonic chicken cells have telomerase activity which, after serial culture passages, is downregulated and cells growth arrest at about 35 PD. At senescence, these cells exhibit mean telomere sizes of about 5 kb (Venkatesan and Price 1998). This value is also similar to the one observed in human senescent cell cultures (5-6 kb). However, this growth arrest could have been driven by inadequate growth conditions leading to senescence so the critical experiment to establish a telomere-based senescence would be

to verify that one could immortalize these cells through ectopic telomerase expression (Forsyth, Wright et al. 2002).

Adult blood cell average telomere lengths in two longlived seabirds, the European shag (*Phalacrocorax aristotelis*) and the wandering albatross (*Diomedea exulans*), were 8.4 kb and 9.9 kb respectively. Telomere length in blood cells shortened between the chick stage and adulthood in both species. However, among adults, telomere length was independent of age (Hall, Nasir et al. 2004). Other studies in lesser black-backed gulls (*Larus fuscus*) showed that larger hatchlings had shorter telomere lengths, suggesting that embryonic growth rate could have influenced telomere attrition. It was also observed that males had longer telomeres at hatching than females (Foote 2009). In two long-lived seabirds, the northern and southern giant petrels (*Macronectes* spp.) telomeres were shorter in adults than chicks, but there was also no trend for adult telomere length to decrease with age (Foote 2009). Telomere shortening in erythrocytes was reported in a variety of avian species by comparing erythrocyte and sperm telomere length (Delany, Krupkin et al. 2000; Taylor and Delany 2000). In a study of 18 species of birds, most displayed the Class I, II and III telomeric arrays (Delany, Krupkin et al. 2000). Extremely long arrays, ranging from hundreds of kilobases to 1-2 Mb (Class III) were observed in all but two raptor species, the northern goshawk (*Accipiter gentilis*) and the American bald eagle (*Haliaeetus leucocephalus*). In erythrocytes of zebra finch (*Taeniopygia guttata*), common terns (*Sterna hirundo*), tree swallows (*Tachycineta bicolor*), and Adélie penguins (*Pygoscelis adeliae*) the average TRF length decreases with age (Haussmann, Winkler et al. 2003). Lifespans of these species range from 5-26 years.

Surprisingly, in Leach's storm-petrel (*Oceanodroma leucorhoa*) erythrocytes, TRF length did not decrease but actually increased with age. This species is long-lived, with observed lifespans of 36 years (Haussmann, Winkler et al. 2003). Higher telomerase activities are observed in the Leach's storm-petrel in most tissues studied (intestine, liver, kidney, brain, bone marrow). Across these species and all age-classes, telomerase activity is generally higher in the proliferative tissues than in the post-mitotic tissues. Telomeric shortening per year was higher in species of birds with shorter lifespans than in the species with longer lifespans (Haussmann, Winkler et al. 2003). The short-lived zebra finch and tree swallow sharply down-regulate bone marrow telomerase before adulthood, whereas the long-lived common tern and Leach's storm-petrel express bone marrow telomerase at high levels throughout life that could produce the slower rates of erythrocyte telomere shortening observed. Post-natal telomerase activity is generally absent in the brain, skeletal muscle, kidney and liver in all species, although higher telomerase activity is observed in the skeletal muscle, kidney and brain of hatchling common terns and Leach's storm-petrels than what is reported in chickens. Telomerase profiles in the bone marrow, gonads and intestine are elevated at all stages of life (Haussmann, Winkler et al. 2003). Few cancer rate studies in long-lived bird species are available but a low incidence of cancer has been reported in wild birds, and specifically in long-lived seabirds (Siegfried 1983; Haussmann, Winkler et al. 2007). Damage susceptibility, repair abilities, shelterin proteins (which control the synthesis of telomeric DNA by telomerase) are also likely to be important in determining these telomeric shortening rates.

Telomeric $(TTAGGG)_n$ sequences are abundant in avian microchromosomes (Nanda, Schrama et al. 2002). In studies of Japanese quail (*Coturnix coturnix japonica*) (unpublished results) telomeric repeats are preferentially localized to the 66 microchromosomes (2n=78) (Fig. 1.6.A). A study of the chromosomal distribution of $(TTAGGG)_n$ sequences in 16 bird species representative of seven different orders, showed that several species, in particular the ratites, display $(TTAGGG)_n$ hybridization signals in interstitial and centromeric regions of their macrochromosomes. The microchromosomes of most species seem to be enriched with $(TTAGGG)_n$ sequences, displaying heterogeneous hybridization patterns, and it has been proposed that this high density of $(TTAGGG)_n$ repeats plays an important role in the exceptionally high meiotic recombination rates of avian microchromosomes (Nanda, Schrama et al. 2002). However, other avian reports claim otherwise (Galkina, Lukina et al. 2005).

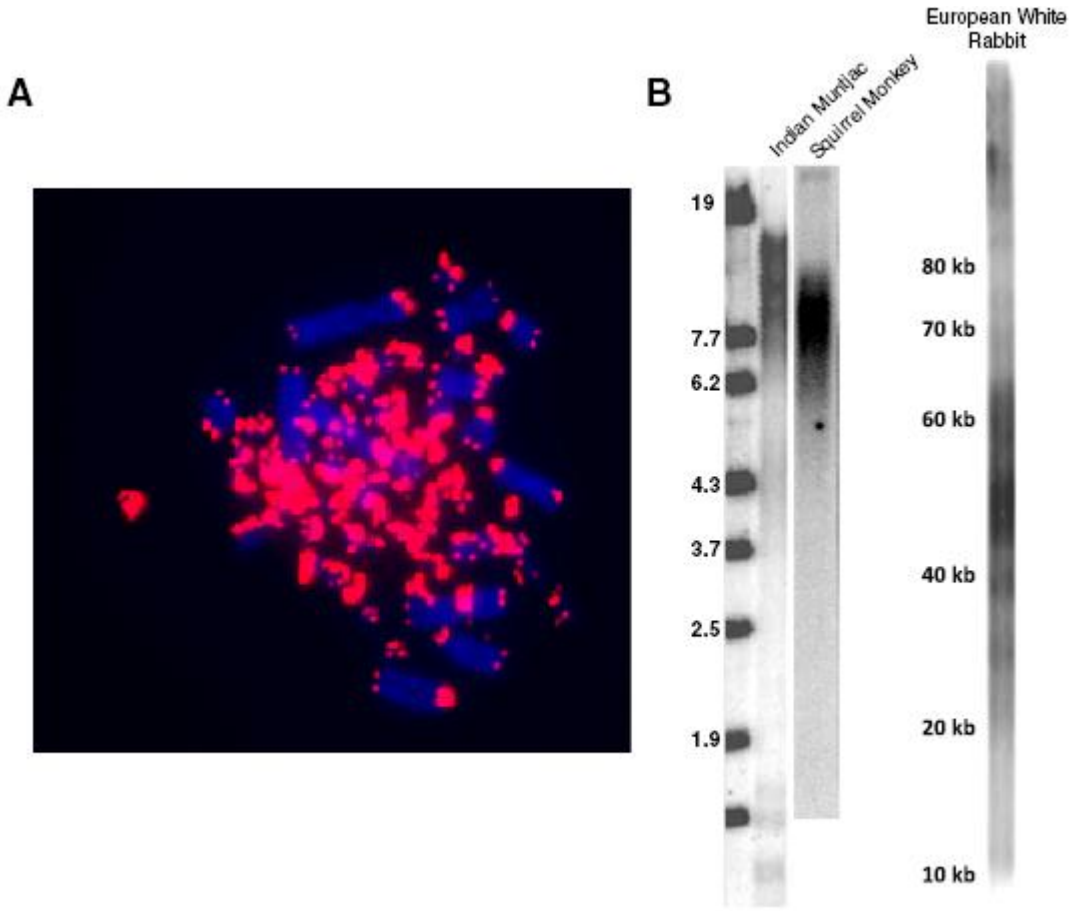


Figure 1.6. Telomeres in vertebrates. A. Quail microchromosomes contain abundant telomeric sequences. Approximately 66 of the 78 quail chromosomes are 7-23 Mb microchromosomes. In situ hybridization using a probe for telomeric repeats reveals that most of the telomeric signal is coming from the small microchromosomes. B. Diversity of mammalian telomere sizes. Primates (squirrel monkey) and Artiodactyls (Indian Munjac, a small barking deer) have small human-like telomeres of less than 20 kb while lagomorphs (European white rabbit) have much longer ~50 kb telomeres.

3.3.2.5 Mammals (Fig. 1.5.k)

Telomere-based replicative senescence is thought to function as a potent mechanism of tumor protection in humans (Lansdorp 2009). It is becoming increasingly evident that many other mammalian species do not use telomeres in this way. For example, there is very good evidence suggesting that mice do not use telomere shortening to produce replicative aging.

Laboratory rodents have extremely long and polymorphic telomeres (25-150 Kb) and shortening was not observed during aging in these inbred laboratory strains of rodents (Kipling and Cooke 1990; Starling, Maule et al. 1990). An *In situ* hybridization study estimated that mice telomeres are long (10-80 kb), but smaller than measured by TRF (possibly due to long subtelomeric regions) (Zijlmans, Martens et al. 1997). Other studies in rat (*Rattus norvegicus*) and mouse (*Mus musculus*) hepatocytes also revealed very long average TRF lengths (50 and 40 kb respectively) (Lejnine, Makarov et al. 1995). Telomere shortening observed in the post-mitotic heart and brain telomeres of mice and rats has been attributed to oxidative stress (von Zglinicki 2000; Oh, Taffet et al. 2001; Forsyth, Wright et al. 2002). Telomerase activity has been detected in most murine tissues. In two mouse strains telomerase activity was reported in adult testes, ovary, breast, colon and liver, but was absent in brain, heart, stomach, muscle and skin (Prowse and Greider 1995; Coviello-McLaughlin and Prowse 1997; Forsyth, Wright et al. 2002). mTERT protein is only found in telomerase positive tissues, but the finding of mTERT mRNA in all tissues (including telomerase negative ones), suggests a quiescent state rather than lack of telomerase competency (Martin-Rivera, Herrera et al. 1998).

Mechanisms of alternate splicing triggered by quiescence may be responsible for the lack of telomerase expression (Martin-Rivera, Herrera et al. 1998; Forsyth, Wright et al. 2002).

The growth crisis that occurs in mouse cultures after 10-15 doublings has been called senescence and was long considered equivalent to the replicative senescence observed in human cultures. Studies of the telomerase negative $mTR^{-/-}$ mouse demonstrate that this growth arrest is not due to telomere shortening and does not limit tumor growth (Blasco, Lee et al. 1997). $mTR^{-/-}$ mouse cells reportedly escape from this growth arrest as frequently as wild-type mice and can continue to divide for at least 200-300 PD (Blasco, Lee et al. 1997). Inadequate culture conditions and diverse environmental stresses can activate growth inhibitory genes due to a process termed stasis (stress or aberrant signaling induced senescence) (Shay and Wright 2004). Senescence in mouse culture occurs as part of a stress response due to inadequate growth conditions similar to reports in some types of human cells. Human skin keratinocytes grown in defined media suffered from p16/RB mediated growth arrest but this could be prevented by growing cells on appropriate feeder layers (Ramirez, Morales et al. 2001). There are now several reports showing that rodent cells have an indefinite replication capacity given proper growth conditions (Loo, Fuquay et al. 1987; Mathon, Malcolm et al. 2001). MEFs (mouse embryo fibroblasts) from mice defective in DNA repair factors such as Ku80, ATM (mutated in Ataxia Telangiectasis) or BrCA2 (mutated in some breast cancers) growth arrest after only 3-4 PD and exhibit high levels of p53 and $p21^{Cip1}$ (Barlow, Hirotsune et al. 1996; Nussenzweig, Chen et al. 1996; Connor, Bertwistle et al.

1997; Wright and Shay 2000). Since these cells divide adequately *in vivo*, the premature growth arrest observed *in vitro* suggests that conventional culture conditions are probably inducing DNA damage which these mutants are unable to efficiently repair. Ambient oxygen is a major contributor to DNA damage, and one major cause of stasis in mouse cells is oxygen since mouse cells grown under reduced oxygen atmosphere do not seem to exhibit cellular senescence (Parrinello, Samper et al. 2003). Furthermore, the Rb pathway does not appear to be involved in cellular growth arrest in mice, but abrogation of ARF/p53 is sufficient to escape this cell growth barrier (Zalvide and DeCaprio 1995; Wright and Shay 2000).

If the stochastic nature of mutations is taken into account, their number will be the result of the product of both time and pool size. Multiplying the weight and lifespan of humans versus mice, humans may need to be about 100,000 times more resistant to the formation of tumors than rodents. The telomerase knockout mouse $mTR^{-/-}$ still displays a normal frequency of neoplasias suggesting that escaping replicative aging by telomerase activation is not a requirement for murine tumorigenesis and that the involvement of other mechanisms of tumor protection such as cell cycle checkpoints, immune surveillance and cellular/DNA repair are sufficient for tumor protection during the short life of these small sized rodents (Blasco, Lee et al. 1997; Wright and Shay 2000; Forsyth, Wright et al. 2002). Together, these results show that the senescence of mouse cells in culture is not due to telomere shortening, and that telomere based replicative aging is not used as a tumor protection mechanism in laboratory mice. There are examples of wild

rodents such as the Algerian mouse (*Mus spretus*) that display "human sized" telomeres (Coviello-McLaughlin and Prowse 1997), and it has not yet been specifically established whether or not they might the use telomere shortening as a tumor protection mechanism.

Telomerase activity was detected in somatic tissues of 15 rodent species, and long telomere lengths (>30 kb) were observed in most species. The lowest levels of telomerase activity were seen in the largest species tested, beaver and capybara, which (together with guinea-pig and deer mouse) displayed shorter "human-like" telomeres (Seluanov, Chen et al. 2007). These authors suggested that telomerase activity co-evolves inversely with body mass, not lifespan, with larger rodents displaying lower telomerase activities, and that telomere length did not show any correlation with size or lifespan (Seluanov, Chen et al. 2007).

Our current knowledge of the consequences of inadequate growth conditions leads us to conclude that studies claiming that the replicative potential of fibroblasts positively correlates with body mass or longevity need to be re-evaluated (Rohme 1981; Lorenzini, Tresini et al. 2005). The studies of Lorenzini (2005) included the early growth arrest in culture of fibroblasts from smaller, shorter lived species such as rodents (half of the species) and carnivores as an example of telomere-based replicative aging(Lorenzini, Tresini et al. 2005). In fact, studies show that fibroblasts from many of these species, given adequate media and more physiological (2% O_2) growth conditions, can grow for over 100 PD (Guyton and Hall 1966; Wright and Shay 2006).

Telomere biology has been studied in a few domestic and farm animals. In horse (*Equus equus*), telomere shortening was observed in fibroblasts cultured to senescence. No telomerase activity was detected in primary cell cultures, in normal equine tissues or equine benign tumor samples of the sarcoid or papilloma type. In adult donkeys (*Equus asinus*) blood samples, telomeres ranged from 7 to 21 kb and telomere lengths was showed to decrease with lifespan (Argyle, Ellsmore et al. 2003). Sheep (*Ovis aries*) dermal and lung fibroblasts have a finite lifespan in culture, after which the cells growth arrest. Terminal restriction fragment lengths from sheep tissues reveal "human-like" telomere lengths (9-23 kb). Telomerase activity is found in the testis but suppressed in somatic tissues. Similarly to humans, senescent sheep skin fibroblasts have increased levels of p53 and $p21^{WAF1}$ compared to young cells (Davisa, Skinnera et al. 2005). Pigs (*Sus scrofa*) also seem to display replicative aging (Pathak, Multani et al. 2000). Among Carnivores, several breeds of dog (*Canis lupus familiaris*) show heterogeneity in telomere lengths in their somatic tissues. Average telomeres range between 12 and 23 kb. Telomerase activity was low or absent in normal somatic tissues and was detected in testis and tumor tissues. Canine soft tissue sarcomas with mean TRFs of 22 and 18 kb have been reported (Nasir, Devliny et al. 2001). In tissues obtained from 2 domestic shorthair cats (*Felis catus*) mean TRF values ranged 5 to 26 kb and there was significant telomeric attrition with increasing age of cats. The same study did not detect telomerase activity in normal tissues (McKevitt, Nasir et al. 2003). Another report in cats showed that average telomere lengths of lymphocytes and granulocytes, analyzed by fluorescence *in situ* hybridization and flow cytometry (Flow FISH), are 5- to 10- fold longer than in

humans. However, much higher telomeric shortening rates are observed both *in vivo* and *in vitro* (500 bp/PD in T cells), suggesting that this shortening might not be caused by the end replication problem but by other mechanisms (Brummendorf, Mak et al. 2002).

During the last several years I have been investigating the role of telomeres and replicative aging in most orders of the mammalian radiation. We have shown that, as humans, other primates also exhibit replicative aging (Steinert, White et al. 2002). In skin fibroblasts from the "Old World" primates [rhesus monkey (*Macaca mulatta*), orangutan (*Pongo pygmaeus*), and pigmy chimpanzee (*Pan paniscus*)] and "New World" primates [spider monkey (*Ateles geoffroyi*) and squirrel monkey (*Saimiri sciureus*)] telomere shortening limits replicative capacity. Human telomerase expression in anthropoid fibroblasts is able to produce telomere elongation and the extension of their *in vitro* lifespan (Steinert, White et al. 2002). A longitudinal study in leukocytes from outbred newborn baboons (*Papio hamadryas cynocephalus*) reveals heterogeneity in telomere length, with two animals having longer telomeres at birth (≈25–28 kb) compared to two other animals (≈13-15 kb) (Baerlocher, Rice et al. 2007). The same Flow-FISH study reported a fast telomere length shortening of about 2-3 kb during the first year of life. However, after the first 50-70 weeks, telomere length appeared to stabilize, leading to the hypothesis that baboons hematopoietic stem cells switch from a fast expansion stage to a phase with significantly lower turnover rate (Baerlocher, Rice et al. 2007). In contrast to the rigorous control of replicative aging by telomere shortening conserved among anthropoid primates, barriers to immortalization were reduced in the prosimian ring-

tailed lemur (*Lemur catta*). Lemur cells have both long and short telomeres. Following ~150 days of senescence a subset of lemur cells showing reduced chromosome number overgrew the cultures without activation of telomerase and displayed increased apoptosis (Steinert, White et al. 2002). The lack of telomerase and the presence of large amounts of extrachromosomal telomere sequences indicates that they had spontaneously activated the ALT mechanism, something that essentially never happens in human cells.

Among Artiodactyla, we studied the small Asian barking deer, the Indian Muntjac (*Muntiacus muntjak*). This species is an ideal model to study telomere biology since it has the fewest number of diploid chromosomes of all mammals with only six chromosomes (1, 2, 3) in the female and seven in the male (1, 2, 3 + X) (Wurster and Benirschke 1970). We observed that Indian muntjac skin fibroblasts growth arrested at PD 89 and that human TERT expression can immortalize them (Zou, Yi et al. 2002). Approaching senescence, the telomeric ends gradually became FISH signal-free and chromosome abnormalities increased dramatically. This species is an excellent candidate as a telomere-based replicative senescence model for human cells (Zou, Yi et al. 2002). In Indian Muntjac we also observed that interstitial telomere sequences coincided with fragile sites, suggesting that these remnants of chromosome fusion events might play a role in genome instability (Zou, Yi et al. 2002). These intrachromosomal TTAGGG sequence sites are known to be fragile "hot spots" prone to breakage and recombination in the Armenian hamster (*Cricetulus migratorius*) and Chinese hamster (*Cricetulus griseus*) (Baxter, Greizerstein et al. 1993; Day, Limoli et al. 1998; Zou, Yi et al. 2002) and are thought to be involved in the process of karyotype evolution during speciation

due to Robertsonian fusions (Meyne, Baker et al. 1990; Rocco, Costagliola et al. 2001).

I have now analyzed fibroblasts from skin and other organs from over 60 animals representing most orders of the mammalian radiation (next section). I addressed the question of whether there was a relationship between senescence in cell culture, cellular telomerase expression, telomere size, telomere shortening rates, ability of hTERT to immortalize versus longevity or the respective damage susceptibility and repair abilities in different species. My results show that the telomere-based tumor protection mechanism has deep roots in the mammalian evolutionary tree. However, there is widespread presence of animals with long "mouse sized" telomeres indicating that there are likely trade-offs between repressing telomerase/having short telomeres to count cell divisions/tumor protection or maintaining telomerase activity and having very long telomeres. Species from the orders Cetacea, Artiodactyla, Perissodactyla, Hyracoidea, Proboscidea and Xenarthra exhibit telomere-based cellular replicative aging. One of the species that displays replicative aging is the bowhead whale (*Balaena mysticetus*). The presence of 19th century stone harpoon points and changes in aspartic acid levels in eye lenses indicates at least one bowhead whale lived approximately 211 years (between 177 to 240 years) (George, Bada et al. 1999). The oldest known ages for other whales are 100 years for a blue whale and 114 for a fin whale (based on counting of waxy laminates on the inner ear plug). The challenges of living in Arctic waters may nurture slow growth and long life (George, Bada et al. 1999; Rozell 2001).

However, most species from important orders such as Rodentia, Chiroptera, Insectivora, and Macroscelidea, do not exhibit replicative aging, with their cells maintaining good telomerase activity and having very long telomeres. We find that Lagomorpha cells, although mostly telomerase negative, do not growth arrest in culture due to their extremely long telomeric arrays (Fig. 1.6.B). Endogenous telomerase activity is present in the North American pika (*Ochotona princes*). These data suggest it is unlikely that lagomorphs use telomere shortening and replicative senescence as a tumor protective mechanism (Forsyth, Elder et al. 2005). Results are less clear in Carnivora where more heterogeneity is observed. Moreover, non-placental mammalian orders such as Marsupials and Monotremata show evidence of alternative mechanisms of telomere maintenance including the presence of restriction enzyme recognition sites intercalated between the telomeric $(TTAGGG)_n$ sequences. Marsupials are particularly interesting species, not only due to their placement at the very base of the mammalian evolutionary tree, but also for their low metabolic rates (70-80 % of similar sized eutherians) (Austad 1997). According to the rate of living theory they should be longer lived, but in fact, they are short-lived for their size (Austad 1997).

There may be trade-offs between the advantages of repressing telomerase/having short telomeres to count cell divisions/tumor protection and the advantages of maintaining telomerase activity and having very long telomeres. We also observe that species using replicative aging tend to have longer lifespans and higher adult body weights, and that telomeric patterns tend to be conserved within evolutionary blocks (e.g.

the bulk of rodents and nearby species have very long telomeres, although individual species such as the deer mouse can have short telomeres).

The presence of several large clades of species having long telomeres interspersed with large clades having short telomeres suggests that the switch between these telomeric strategies has happened several times, reinforcing the concept that there must be advantages/tradeoffs between each pattern of telomere biology. One working hypothesis is that if a long-lived animal that used replicative aging as a tumor-protection mechanism occupied a short-lived niche, it would then be investing excess resources in tumor protection. Since it would already have adequate DNA-repair/immune surveillance/etc. mechanisms to prevent tumors during its short lifespan without the additional barrier of replicative aging, it might abandon replicative aging if there was a compensatory advantage. One such advantage might come from the ability to reduce levels of oxidative protection. Telomeres are very sensitive to oxidative damage, both because triplet Gs are a preferential target for free radicals (Oikawa and Kawanishi 1999) and there are triplet Gs within every TTAGGG repeat, and because the proteins/structures that hide the end of the chromosome from being recognized as a double strand break also reduce the efficiency with which oxidative damage is recognized and repaired (von Zglinicki 2002). Having very long telomeres would permit losses of large telomeric repeats due to oxidative damage without denuding the telomere, and not repressing telomerase would permit the repair and elongation of excessively shortened telomeres. Thus abandoning small telomeres that shortened in a well-regulated fashion to count cell divisions to serve

as a tumor protection mechanism in favor of very large telomeres and not repressing telomerase would have the potential advantage of permitting a reduction in the energy invested in oxidative damage protection. There is a good correlation between having long telomeres and telomerase activity and the rapid appearance of culture stasis, indicating that in general species using the long-telomere strategy are sensitive to the stresses of the tissue culture environment. Preliminary studies in members of several orders of mammals suggest that a much greater correlation exists between resistance to some inducers of oxidative stress and the telomere strategy of the group than between resistance and lifespan (Kapahi, Boulton et al. 1999; Lorenz, Saretzki et al. 2001; Murakami, Salmon et al. 2003; Parrinello, Samper et al. 2003; Harper, Salmon et al. 2007). Species that use replicative aging seem to have better cellular protection / repair mechanisms to some types of stress than species that are telomerase positive that do not use this tumor protection mechanism.

The results of this ongoing study (presented in the next chapter) are providing insights into the role of mammalian telomeres as tumor protectors, novel ALT mechanisms, telomere regulatory strategies and the role of replicative senescence in human aging. The results of these experiments should help to clarify the biological importance and evolutionary flexibility of telomere-based replicative aging.

4. Animal Cloning

An initial report comparing telomere lengths of sheep derived by natural mating and nuclear transfer suggested that somatic telomeres decrease in length with age, and that Dolly, derived by the transfer of a 6 year-old adult somatic nucleus, exhibited diminished telomere lengths (Shiels, Kind et al. 1999). This was proposed to limit the utility of cloning for replacement of cells and tissue for human transplantation. However, the reported difference was well within the normal TRF variation range. Given the activation of telomerase at the blastocyst stage, reprogramming of the adult nucleus is likely to involve reactivation of telomerase and resetting of the telomeres to normal levels (Forsyth, Wright et al. 2002). In fact, subsequent studies have shown that aged adult fibroblasts were suitable as nuclear donors (Kubota, Yamakuchi et al. 2000). In cloned calves derived from senescent donors, somatic cell nuclear transfer prolonged the replicative lifespan of senescent cells and telomeres were extended beyond those of newborn (2 weeks old) and age-matched control animals (Lanza, Cibelli et al. 2000). Moreover, telomerase activity has been found in the blastocysts of post-clonal embryos, independently of the age of the nuclear donor (Betts, Bordignon et al. 2001; Forsyth, Wright et al. 2002). The ability of nuclear transfer to restore somatic cells to a phenotypically young state has important implications for agriculture and medicine (Lanza, Cibelli et al. 2000).

5. Conclusion

Telomerase plays a vital role in chromosomal maintenance and stability in unicellular and multicellular organisms. In invertebrates, Fish, Amphibian, and Reptiles persistent telomerase activity in somatic tissues also allows the maintenance of the incredible regenerative potentials of these species. The lack of telomerase repression in poikylotherms suggests that these animals do not use replicative aging, and that replicative aging may have evolved to provide an additional barrier to tumor protection only under the additional mutational load that occurs in eutherians. In birds and many mammals, the efficient tissue repression of telomerase suggests that they might use replicative aging as a tumor protection mechanism, similar to humans, while other mammals appear to have adopted another telomere strategy that has abandoned replicative aging. The link between replicative senescence and aging remains controversial but it has been established in some age-related human diseases (Blasco 2007). Also, the role of telomeres and telomerase regulation in embryonic and adult stem cells has placed telomerase "back in the game" of this exploding field of stem cell biology (Blasco 2007). The understanding of telomere biology has already led to the development of several telomerase inhibitor drugs that are in advanced clinical trials and can soon be part of the human chemotherapy cocktails (Dikmen, Gellert et al. 2005). Telomerase activators which can potently lead to increased tissue regeneration are already commercialized in the United States (http://www.tasciences.com/index.html). The recent addition of more species to the genome and protein databases, will allow an

emergence of more in depth studies on the role of the shelterin proteins in telomeric regulation during development and aging in many multicellular organisms.

CHAPTER TWO

The Comparative Biology of Mammalian Telomeres

1. Introduction

Telomerase, the enzyme that maintains telomeres, is absent from most adult human somatic cells, producing a progressive telomere shortening that limits the proliferative potential of primary human cell cultures (Shay and Wright 2007). This programmed telomere shortening, replicative aging, functions as a tumor suppressor program that generates a barrier for the outgrowth of tumors. Remarkably, this telomere tumor suppressor program is not conserved in laboratory rats and mice, which have long telomeres and constitutive telomerase (Sherr and DePinho 2000; Wright and Shay 2000). The present study examines over 60 mammalian species to determine the phylogenetic distribution of the telomere tumor suppressor pathway. Phylogeny based statistical analysis demonstrates that telomere length inversely correlates with lifespan but not body size, while telomerase expression inversely correlates with body size but not lifespan. The ancestral mammalian phenotype was determined to have short telomeres and repressed telomerase. At least 5-7 independent times in different orders smaller, shorter lived species changed to having long telomeres and expressing telomerase, suggesting tradeoffs between the advantages and drawbacks of using replicative aging as a tumor suppression mechanism. I show that one advantage is consistent with reducing the

energetic/cellular costs of specific oxidative protection mechanism needed to maintain short telomeres. I propose that the telomere tumor suppressor pathway represents an initial adaptation to the increased mutational load of homeothermy by ancestral mammals, has adaptive advantage in large and long-lived animals, but has been abandoned by many species. These observations resolve a longstanding confusion about the use of telomeres in humans and mice, support a role for telomere length in limiting lifespan, provide a critical framework for interpreting studies of the role of oxidative protection in the biology of aging, and identify which mammals can be used as appropriate model organisms for the study of the role of telomeres in human cancer and aging.

2. Materials and Methods

2.1. Cell Culture

Fibroblasts from the skin, kidney, lung or cornea of various adult mammalian species were grown in a variety of media (Supplementary Fig. S1). All cells were grown at 37^{o}C in 5% CO2 and low oxygen conditions (2-5% O2) (Wright and Shay 2006) except as noted in Supplementary Fig. S1.

Bowhead whale, naked mole rat and Virginia opossum were grown at 33^{o}C. Echidna was grown at 28^{o}C. Bowhead whale, horse and zebra cells were grown in collagen coated dishes. All cells were passaged weekly at about 70-80 % confluence.

2.2. Viral transfections

Some cells rapidly entered stasis, a growth arrest due to inadequate growth conditions. In order to force these cells to proliferate we overexpressed SV40 Large-T antigen to block cell cycle checkpoints, cyclin-dependent kinase 4 (Cdk4), or human papillomavirus (HPV) E6/E7. In cases where the cells were non-dividing at arrival, they were first infected with adenovirus expressing SV40 LgT antigen to initiate cell division, and then infected with retroviral vectors to produce stable expression. Stable infection with retrovirus expressing SV40 LgT antigen was performed 72 h after adenoviral infection. Stable expression of the human telomerase protein catalytic component (hTERT) used the retroviral vector pBabepurohTERT (Ouellette, Aisner et al. 1999).

2.3. Telomere length analysis

The mean telomere length was determined using terminal restriction fragment (TRF) analysis (Herbert, Shay et al. 2003). Genomic DNA was digested with one or a mixture of six enzymes (HaeIII, AluI, Hinf I, MspI, RsaI and CfoI) and resolved on 0.7% agarose gels. High molecular weight DNA was resolved on a 1% agarose gel using field inversion gel electrophoresis (1-50 Kb) performed with the FIGE-Mapper (BioRad). After denaturing and drying of the gels, they were hybridized with telomere high specific activity probe (4h at 42 ^{o}C). The gels were washed with 2x SSC (15 min), 0.1x SSC/0.1%SDS (2x 10 min) and 1x SSC (5 min). After exposing a phosphor screen it was scanned with a Storm 860 PhosphorImager system and visualized with ImageQuant

software (Molecular Dynamics). The Mean Telomere Length was calculated using the TELORUN program.

Telomere size varies by 3-4 kb between different human donors in addition to decreasing with donor age (e.g., (Benetos, Okuda et al. 2001)). Since a single culture from an animal of undetermined age was analyzed, telomere size was estimated (accurate within a few kb) rather than calculated to emphasize the limits of this analysis (i.e., only >5 kb differences are significant).

2.4. Non-denaturing in-gel hybridization to detect telomere DNA

In some cases it was difficult to distinguish telomeres from abundant internal telomeric repeats. The true telomeric signal was then determined on native gels by hybridizing ds genomic DNA to a C-rich telomeric probe that would only anneal to the single-stranded G-rich telomeric overhang.

After digesting 5 μM of genomic DNA with the 6 restriction enzyme mix, DNA was annealed to 5 fmol of C-rich oligo (GTU3) in 100mM salt, o/n, at room temperature. Electrophoresis was performed at 50V at 4 ^{o}C for about 40h. After denaturing the gel, it was dried upside down to a N+ membrane (Hybond). The denatured probe melts off the telomeric overhangs, migrates out of the gel and bounds to the membrane. After neutralization, the nylon membrane was air dried, exposed to a phosphor screen and scanned with a Storm 860 PhosphorImager system to reveal the location of the telomere G-overhangs. The gel was then probed with TRF probe as described in the previous section. The comparison of the membrane and gel allows for the location or the terminal

telomeres containing single stranded G-overhangs versus the exposed gel with contains additional interstitial telomeric sequences.

2.5. Telomerase Activity measurement

Cell telomerase activity was measured using the telomeric repeat amplification protocol (TRAP) (Herbert, Shay et al. 2003), and activity expressed with respect to the reference human lung adenocarcinoma line H1299. 100,000 cells were lised on NP-40 buffer. The extension products of telomerase were amplified by a three-step PCR (95 ^{o}C for 30s, 52 ^{o}C for 30 s, 72 ^{o}C for 30 sec) for 23 cycles of PCR in the presence of Cy5-labeled TS primer (Integrated DNA Technologies Inc.) and resolved on a 10% nondenaturing acrylamide gel (Bio-Rad). The Cy-5 fluorescence was measured with direct scanning of the gel using a 650 nm filter on a typhoon scanner system. Telomerase activity was calculated as the ratio of the sum of the intensities of the telomerase ladder (6-bp ladder) to the intensity of the 36-bp internal standard control using ImageQuant software (Molecular Dynamics).

It is important to note that *in vivo* telomerase is expressed during embryogenesis in humans and presumably all mammals, and that the expression of telomerase in cells cultured from adult animals is being interpreted as a reflection of the regulation of telomerase in adult somatic cells that are not stem cells.

Tissue biopsies often show weak telomerase activity when first put in culture that disappears within the first few doublings. Whether this represents the presence of stem cells that are overgrown by fibroblasts/more differentiated cells or simply a down-

regulation of telomerase is unknown. Telomerase activity was quantitated from the activity sustained for more than a few doublings in culture.

2.6. Metaphase spread preparation.

Cultured cells were plated at low density for 3 days and incubated with 10 ng/ml colchemid (Gibco) for 12 h. After trypsinization the cells were incubated in hypotonic 0.075M KCL solution at 37^{o}C for 30 minutes, the cells were then fixed with three methanol:acetic acid (3:1) washes; resuspended again in methanol:acetic acid (3:1), dropped onto slides and dried overnight. The remaining cell pellets were stored at -20^{o}C.

2.7. Fluorescence in Situ hybridization (FISH) Analysis

Metaphase spreads were hybridized to 3'-Cy3 or Cy5 conjugated $(CCCTAA)_3$ 2'-deoxyoligonucleotide $N_{3'}$-$P_{5'}$ phosphoramidate telomeric probes (kindly provided by Geron Corp.) as described (Zou, Yi et al. 2002).

One to three day old slides were rehydrated in 1x PBS (pH 7.5) for 15 minutes at room temperature and fixed in 4% formaldehyde in 1x PBS (pH 7.5) for 2 minutes. After three 5 minutes washes with 1x PBS, the slides were treated with pepsin solution (1mg/ml pepsin, pH 2.0) at 37^{o}C for 10 minutes and washed twice for 2 minutes in 1x PBS. Slides were again fixed in the formaldehyde solution for 2 minutes and washed three times in 1x PBS. After dehydration with a 2 minutes serial incubation in 70, 90 and 100% ethanol the slides were air dried and incubated with a hybridization mixture (20µl) containing 3'-Cy3 or Cy5 conjugated $(CCCTAA)_3$ 2'-deoxyoligonucleotide $N_{3'}$-$P_{5'}$

phosphoramidate telomeric probes (kindly provided by Geron Corp.), 70% formamide, 0.25% (w/v) blocking reagent (Roche), and 5mM $MgCl_2$ in 10mM Tris (pH 7.2). After overlay with coverslip, samples were denatured for 10 minutes at 80^{o}C, after which they were annealed for 2 h at room temperature in a humidified chamber. Unbound probe was removed with two 15 minutes washes with 70 % formamide, 0.1 % BSA and 10 mM Tris (pH 7.2). Slides were then washed three times for 5 minutes with 0.1 M Tris, 0.15 M NaCl and 0.008% Tween 20. After a 2 minute ethanol series dehydration, the slides were air dried in the dark overnight. The following day the slides were reprobed for 2 h with 3'-Cy3-conjugated $(GGGATT)_3$ probe and then submitted to the same washes and dehydration procedure. The following day the slides were counterstained in the dark with Vectashield with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.). Metaphase images were visualized and captured using a Zeiss Axiovert 200M inverted microscope (100x/1,40 Plan-Apochromat oil-immersion objective) equipped with precision DAPI/Cy3/FITC band pass filters and analyzed with Axiovision 4.5 software.

2.8. Cytotoxicity Assay

Thawed cells were grown for 11 days then plated at 20,000 cells/100 µl/well in 96 well plates. After a 40 h recovery, 100 µl of RPMI-1640 media containing dilutions of *tert*-Butyl hydroperoxide (C4H10O2) or sodium arsenite (NaAsO2) were added for 4 h at 37^{o}C. After three washes with D-PBS, each well was incubated for 4h at 37^{o}C with 100 µl MTT (Sigma) diluted in RPMI-1640 (with 25mM Hepes and 1 mM NaPyruvate). 200 µl of solubilization solution (10% Triton X-100, 0.1 N HCl, 80 % Isopropanol) were

added to each well and the plate was shaken for 10 minutes. 570 nm absorvance was measured using an automated plate reader (700nm reference wavelength).

2.9. DNA Damage/Repair Assay

Single cell gel electrophoresis was performed according to the Fpg FLARE™ Assay (Trevigen) with modifications(2001). Fibroblasts were grown for 12 days, harvested and washed with ice cold PBS. Cell were imbedded in 0.7 % low melting point agarose and spread over slide sample area. After solidification at 4°C for 30 min, cells were lised for 1 h at 4°C after with they were incubated with 1:1 dilution of Formamidopyrimidine-DNA Glycosylase (Fpg). After DNA unwinding for 30' with solution pH 12.1 at R.T., electrophoresis was performed for 20 min at 4°C. Slides were dipped in 70 % ethanol for 5 min and air dried. After staining with CYBR® Green, images were captured using a Zeiss Axiovert 200M inverted microscope (20x/0.3 LD A Plan objective) equipped with FITC band pass filter and analyzed with Axiovision 4.5 software. Determination of the average Olive tail moment of 100 nucleus was performed using Metasystems comet analysis software.

Olive tail moment = (|CGt – CGh|) x DNA/100

CGt= Center of gravity of the tail or body weighted by gray value s

CGh= Center of Gravity of the head weighted by Gray Value s

DNA= Tail or Body DNA

2.10. Criteria for groupings

Telomeres were classified as long if >20 Kb and short if <20 Kb. It has been established that in some cases there is a large resistant region of genomic DNA (the "X" region) that remains attached to the telomere even following digestion with a mixture of 4-base recognition enzymes (Steinert, Shay et al. 2004; Gardner, Kimura et al. 2007). In some cases (e.g., giraffe, Supplementary Fig. S1) the rapid disappearance of the telomeric signal while the telomeres were still apparently very large implies a very large X-region (compare the giraffe signal at PDs 7-39 to 42-61). Telomeres exhibiting this characteristic were scored as short since the actual size of the telomeric repeat region was clearly <20 Kb. Tissue biopsies often show weak telomerase activity when first put in culture that disappears within the first few doublings. Whether this represents the presence of stem cells that are overgrown by fibroblasts/more differentiated cells or simply a down-regulation of telomerase is unknown. Cultures were scored as telomerase positive only if telomerase activity was sustained in culture.

2.11. Statistical analysis

The phylogenetic regression analysis - phylogenetic generalized least squares models (PGLS)

We used a pruned version of Bininda-Edmonds et al's mammal supertree (Bininda-Emonds, Cardillo et al. 2007; Bininda-Emonds, Cardillo et al. 2008), with branch lengths measured by estimated divergence times in millions of years. We conducted simultaneous multiple regression analyses (so all p-values reported are

independent of all other variables in the model), accounting for the shared ancestry as implied by the phylogeny within a maximum likelihood phylogenetic generalized least-squares framework using the computer program *BayesTraits* (Pagel, Meade et al. 2004) and a model of evolution estimated across the whole tree to estimate ancestral states (Organ, Janes et al. 2009). The parameter 'lambda' accounts for the strength of the phylogenetic signal (Pagel; Freckleton, Harvey et al. 2002).

Telomere lengths

We used a maximum likelihood model that yields the most likely ancestral trait value at the root of a give phylogenetic tree under the evolutionary model of Brownian motion. It is also important to account for the phylogenetic signal in the data when estimating ancestral trait for continuous data as if not the estimate can be inflated. As such we estimated the root ancestral state along with the parameter λ, this parameter is also found by maximum likelihood and potentially varies between 0 (no phylogenetic signal; the species can be treated as independent) and 1 (the observed pattern of trait variation among the species is predicted by the phylogeny (Freckleton, Harvey et al. 2002). We found that the ancestral trait value at the root of the placental mammals = 18.59kb, 'λ' = 1 (very strong phylogenetic signal).

Telomerase (repressed or expressed):

The Markov-transition process is the statistical model widely used to describe the evolution of traits that adopt only a finite number of states. It is routinely used in

phylogeny reconstruction and in comparative methods for reconstructing ancestral character states (Pagel 1994). The Markov approach estimates the rates at which a discrete character makes transitions among its possible states as it evolves through time. These rates are sufficient to calculate the most probable states at ancestral nodes of the phylogeny.

Cellular stress resistance

To investigate the potential impact of phylogeny on the relationship between cellular stress resistance and telomere/telomerase, we employed the method of phylogenetically-independent contrasts (Felsenstein 1985; Garland, Harvey et al. 1992). Our phylogeny was largely drawn from Binenda-Edmonds et al. (Bininda-Emonds, Cardillo et al. 2007) supplemented by additional sources as necessary (Adkins, Walton et al. 2003; Asher, Meng et al. 2005; Teeling, Springer et al. 2005; Agnarsson and May-Collado 2008). Branch lengths were measured by estimated divergence times in millions of years largely from Time Tree (http://www.timetree.org/) (Hedges, Dudley et al. 2006) but also from additional sources as necessary.

3. Results and Discussion

3.1. Evolutionary distribution of telomere length, telomerase activity and stasis

Telomerase expression in dividing cultured fibroblasts from adult donors was used to determine the strength of telomerase repression in somatic mesenchymal cells. Telomere length and telomerase expression in culture from 44 and an additional 18 previously studied species are arrayed on a mammalian phylogeny in Figure 2.1 (see supplemental Fig. Sup. 1 for detailed data for each species). Cells cultured under suboptimal conditions (e.g. lack of a micronutrient, 20% oxygen, often unknown factors) frequently exhibit a growth arrest called stasis (stress or aberrant signaling induced senescence)(Drayton and Peters 2002) that is independent of telomere shortening. The presence of an early stasis (within 15 doublings) also provided an additional phenotype. The resistance to the oxidative damage agent sodium arsenite is also shown for selected species and will be discussed later.

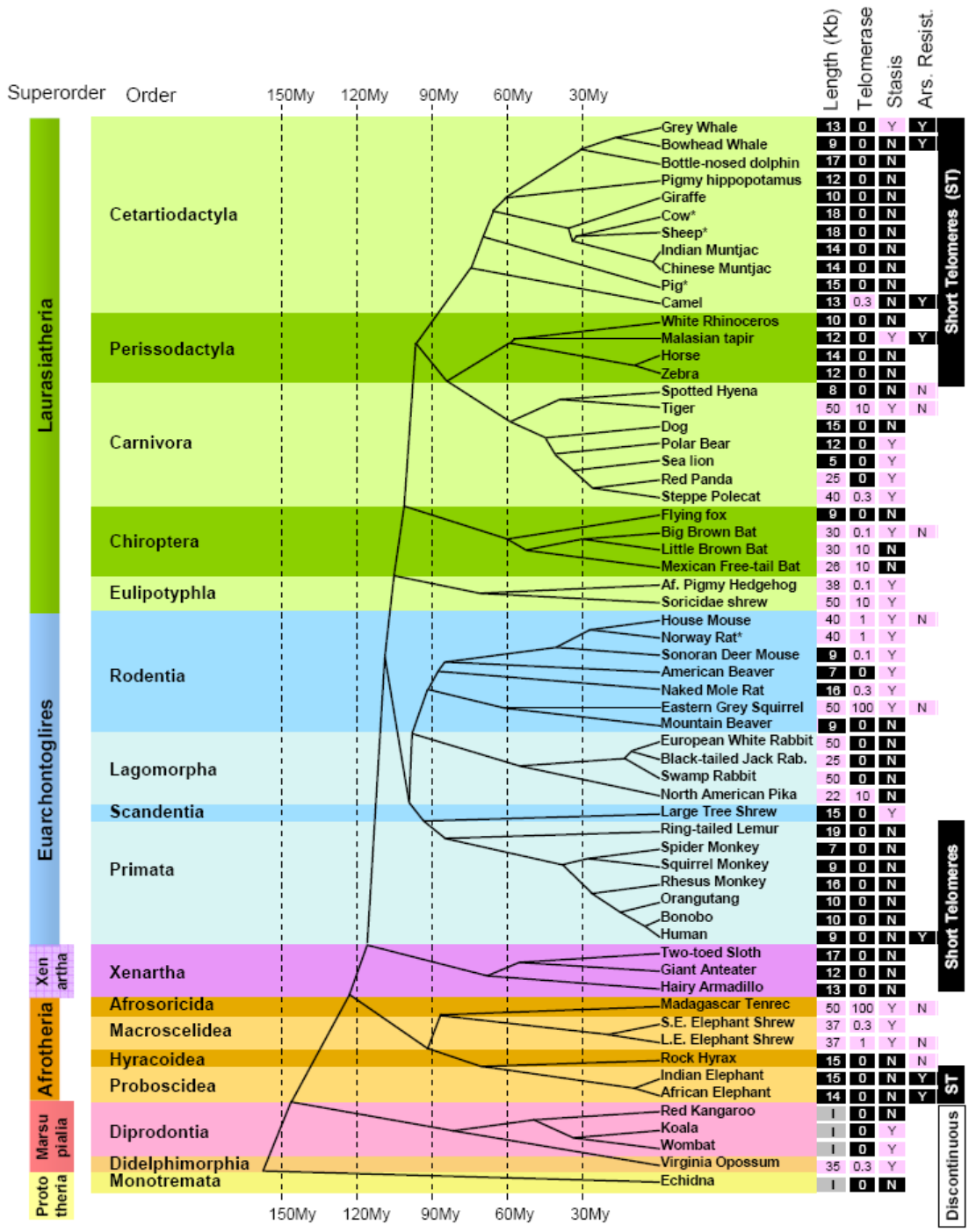


Figure 2.1. Evolutionary distribution of telomere length, telomerase activity and stasis (stress or aberrant signaling induced senescence) in the mammalian evolutionary tree.

Telomere length, telomerase activity and stasis induction run in evolutionary clades. Telomere length was measured by telomere length fragment (TRF) analysis: Values <20 Kb are shaded black while values greater than 20 Kb are shaded pink. Non-placental mammalian orders such as Marsupials and Monotremes show the presence of restriction enzyme recognition sites intercalated between the telomeric $(TTAGGG)_n$ sequences and thus are labeled as being of indeterminate size (I). Telomerase activity was detected with TRAP: Values are expressed as a % of the activity in the reference lung adenocarcinoma tumor line H1299, and shade black if absent and pink if any activity was detected. Stasis: (N) with black shading = cells grew beyond 15 doublings, (Y) with pink shading = cells growth arrested in culture before population doubling 15. Based on these characteristics, some taxonomic orders could be described as exhibiting uniformly short telomere phenotypes or having discontinuous telomeres. Arsenite Resistance: (Y) with black shading = LD90 >20 mM sodium arsenite after 4h exposure, (N) with pink shading = LD90 <5 mM (see Fig. 2.5). Published data on Primates (Steinert, White et al. 2002), Lagomorphs (Forsyth, Elder et al. 2005) and Muntjacs (Zou, Yi et al. 2002) are from my laboratory, and thus are directly comparable. Data from other laboratories are indicated by (*)(Cow (Lanza, Cibelli et al. 2000), Sheep (Cui, Aslam et al. 2002), Pig (Fradiani, Ascenzioni et al. 2004; Oh, Jin et al. 2007), Rat (Mathon, Malcolm et al. 2001)). Scientific names and specific data for each species (scientific name, growth curves, TRF gels, telomerase assays, mass, lifespan) are provided in Supplementary Table 1 and Supplementary Fig. S1. The telomere lengths for Giraffe, Rhinoceros and Anteater are adjusted for a large digestion-resistant subtelomeric region using the rate of

disappearance of the telomeric signal with increased cell doublings (see Supplemental Fig. S1). Cladogram adapted from Bininda-Edmonds (Bininda-Emonds, Cardillo et al. 2007; Bininda-Emonds, Cardillo et al. 2008).

3.2. Lifespan/body mass distribution of telomere strategy species

We examined telomerase expression, telomere length, body size and lifespan using regression models which account for the shared ancestry as implied by the phylogeny within the phylogenetic least-squares framework (PGLS) (Pagel 1999). Telomerase expression was found to significantly contribute to body size ($p=0.0082$), but showed no independent effects on lifespan ($P=0.34$). The same analysis found that telomere length significantly contributed to lifespan ($P=0.0032$) over and above what we would expect from body size alone, but that there was no independent association of telomere length with size ($p=0.71$) (Figures 2.2 and 2.3).

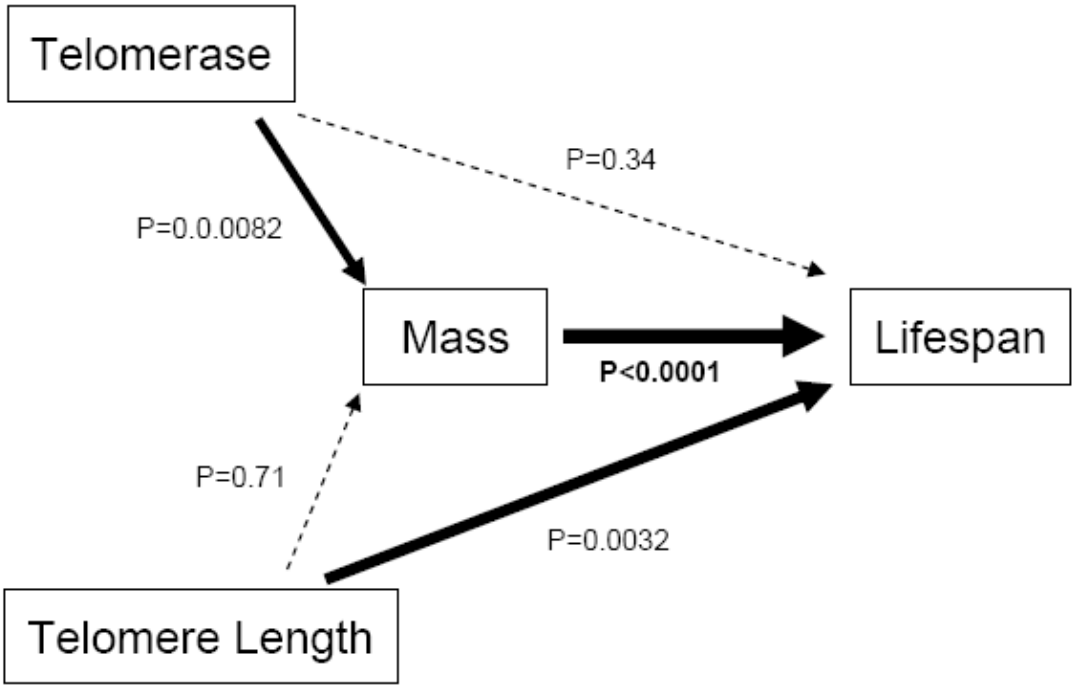


Figure 2.2. Relationship of telomeres and telomerase to mass and lifespan.

Dark arrows indicate significance while dotted arrows show lack of significance. Phylogenetic least squares framework (PLGS) analysis demonstrates that telomerase expression inversely coevolves with increased mass while telomere length inversely correlates with increased lifespan.

In order to use the full power of regulated replicative aging as a tumor suppression mechanism one needs to have both short telomeres and repress telomerase. These results suggest that decreased levels of expression of telomerase alone can nonetheless confer advantages as the number of cells in the body increase (with size).

This could be due to the ability of telomerase to repair telomeres that have suffered catastrophic deletion events (for example, expression of telomerase might allow a premalignant cell lacking cell cycle checkpoints to survive a deletion resulting from a stalled replication fork at telomeres (Sfeir, Kosiyatrakul et al. 2009)). Alternatively, telomerase expression could have additional effects unrelated to maintaining telomeres (Park, Venteicher et al. 2009). There is a well established general correlation of body size and lifespan. Once the number of cells has reached a certain level, the independent association of short telomere lengths with increased lifespan suggests that the full establishment of replicative aging is required in order to suppress tumor formation over longer periods of time.

The data for the relationships among the variables analyzed in the regressions are shown in Figure 2.3. These same qualitative forms are also found separately within at least four orders (Carnivora, Chiroptera, Rodentia, Lagomorpha) that show a wide variation in telomere length and telomerase expression, demonstrating that they have evolved multiple times in the class mammalia (see Supplemental Table S2). Previous observations concluded that telomerase expression *in vivo* decreases with increasing body size in rodents(Seluanov, Chen et al. 2007; Gorbunova and Seluanov 2008), but that there was no relationship with lifespan for either telomerase activity or telomere length. The present results demonstrate that in a broad mammalian analysis telomere length does coevolve with lifespan.

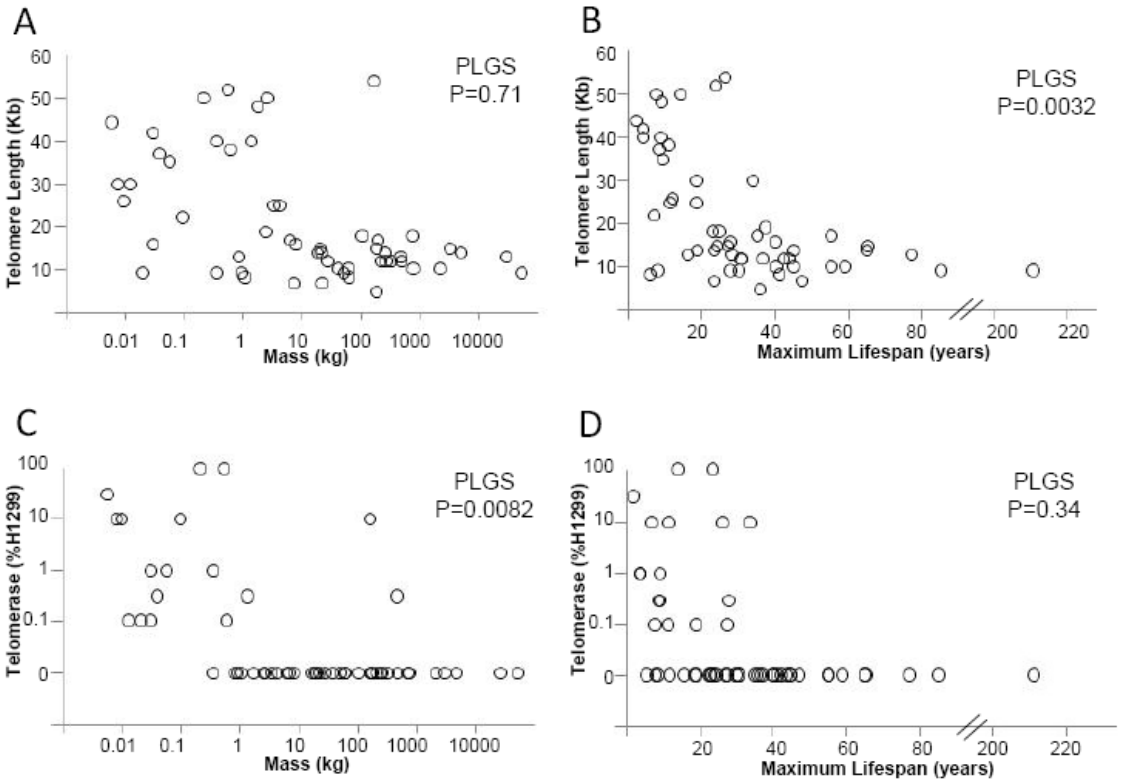


Figure 2.3. Telomere length, telomerase, lifespan and body mass distributions.

Telomere length (A and B) or Telomerase (C and D) vs log body mass (A and C) or lifespan (B and D). The probability of a significant relationship is indicated by the PLGS analysis, which takes into account phylogenetic relationships (which are not indicated in the figure). Telomerase is significantly inversely related to mass while telomere length is inversely correlated with lifespan.

3.3. Ancestral phenotypes

The ancestral telomere length at the base of the placental mammals was reconstructed using maximum likelihood models that yield the most likely value at the root of a given phylogenetic tree under the evolutionary model of Brownian motion, along with the parameter "λ", which measures the strength of the phylogenetic signal (Freckleton, Harvey et al. 2002; Pagel, Meade et al. 2004; Organ, Janes et al. 2009). The ancestral state was determined to be 18.6 kb with a λ equal to 1, indicating a very strong phylogenetic signal. A Markov-transition approach (Pagel, Meade et al. 2004) was used to estimate the ancestral state for the expression of telomerase. The probability that ancestral placental mammals repressed telomerase was calculated to be high (1) compared to the probability that it was expressed (0). These results remained qualitatively the same even if each order was analyzed separately (Supplementary Table S2). The transition rate from expressed to repressed was estimated to be close to zero, meaning that only transitions from repressed to expressed occurred. Although only limited data is available, a broad range of poikylothermic aquatic species from echinoderms to cartilaginous and bony fishes have short telomeres and express telomerase in many of their tissues (see chapter 1 and (Elmore, Norris et al. 2008)). The presence of short telomeres with repressed telomerase as the ancestral placental mammalian phenotype suggests that one of the early adaptations to becoming homeothermic with its accompanying increased mutational load was the repression of telomerase in adult somatic cells while having short telomeres, thus initiating telomere shortening as a tumor-protection mechanism.

3.4. Non-placental mammal telomere grouping

The ancestral mammal was probably most similar to the non-placental mammals, but we were not able to unambiguously determine their telomere phenotype. The sequence TTAGGG lacks restriction sites. Telomere length is normally determined by digesting genomic DNA with a mixture of four-base recognition restriction enzymes to remove sequence-diverse DNA from the internal (centromeric) side of the telomere, and then measuring the size of the telomeres on agarose gels. The non-placental mammals contained telomeres in which kilobase long stretches of telomeric repeats were interrupted by DNA containing restriction sites. Figure 2.4 compares the size distribution of telomeres from species from orders with uniformly short telomeres, examples of long telomeres from orders with variable telomere lengths, and two marsupials. The wombat and koala telomeres appear to be less than 2 kb long when digested with our routine mixture of six enzymes, but exhibit completely different patterns when digested with individual enzymes. The size of their telomeres varied from long to very short depending on which 4-base recognition restriction enzyme was used to digest the DNA, and thus the size of their telomeres could not be determined. Ribosomal DNA sequences have been found interspersed with telomeric repeats at the acrosomal telomeres of one but not other Sorex species (Zhdanova, Minina et al. 2007). The nature of the interspersed sequences in the non-placental mammals and whether they reflect past recombination/insertion events or an ongoing process involved in telomere maintenance remains to be determined. The lack of telomerase expression by koala cells, their growth arrest after only 38 divisions even after blocking cell cycle checkpoint functions, and their immortalization following

the introduction of hTERT (Supplementary Fig. S1) suggests that at least one marsupial is using replicative aging and that only the most terminal stretch of uninterrupted telomere repeats is functioning in this process.

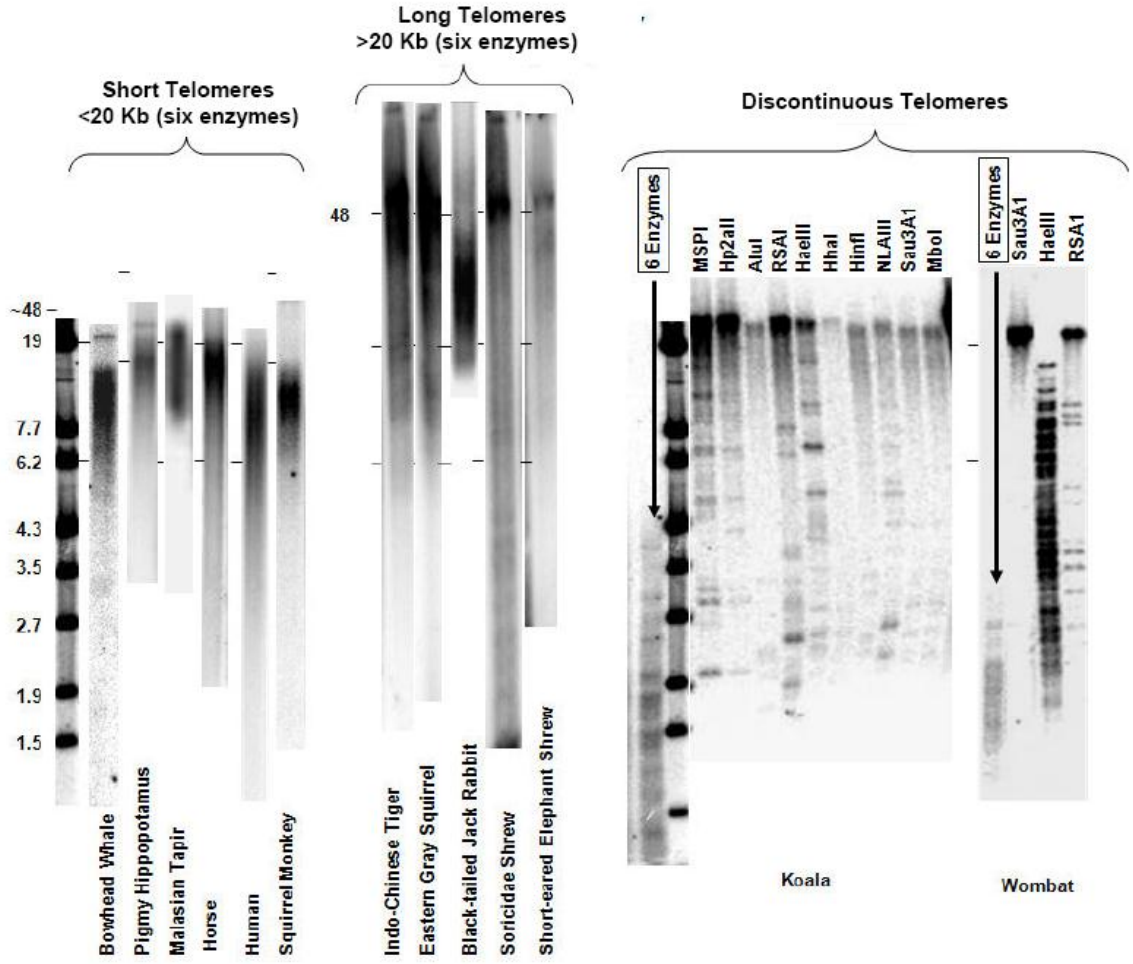


Figure 2.4. Non-placental mammals have discontinuous telomeres.

Digestion of genomic DNA with a mixture of six 4-base restriction enzymes (HaeIII, AluI, HinfI, MspI, RsaI and CfoI) (Telomere Restriction Fragment analysis, TRF) yields telomeres of a variety of sizes, examples of which are shown. Non-placental mammalian

repeats are interrupted by DNA containing restriction sites, so their size depends on which enzyme is used. Dashes corresponding to the 19 and 6.2 kb size markers were digitally fixed to the image for each species, and then the size of each image adjusted so that all the marks aligned to allow a direct visual comparison between the different species. The DNA from species with very long telomeres were analyzed on FIGE gels.

3.5. Evolutionary trade-offs

It is widely believed that ancestral mammals were small. However, most current small species (less than 1 kg) have long telomeres and express telomerase (Fig. 2.3). The phylogenetic analysis suggests that the ancestral mammalian phenotype had short telomeres and repressed telomerase as the initial adaptation to homeothermy, and that the acquisition of long telomeres and not repressing telomerase represents secondary changes that provided adaptive advantage to species that either remained small or which evolved from larger precursors (such as may have occurred in bats (Simmons, Seymour et al. 2008)). These smaller species came to have longer telomeres and express telomerase at least 5-7 independent times in the evolutionary tree. One advantage might reflect the trade-off between the benefits of tumor suppression and the costs of limiting regeneration. Human diseases involving mutations in telomerase cause premature stem cell depletion and a variety of age-related diseases such as sporadic bone marrow failure, dyskeratosis congenita and idiopathic pulmonary fibrosis (Garcia, Wright et al. 2007), contributing to the hypothesis that telomere shortening may contribute to some aspects of human aging. One advantage of not using replicative aging would be an increase in

regenerative capacity. An additional trade-off might involve a higher amount of resources invested in oxidative damage protection in species with short telomeres. Free radicals preferentially damage GGG triplets (Hall, Holmlin et al. 1996; Oikawa and Kawanishi 1999), present every six bp in the mammalian TTAGGG telomeric repeat. Furthermore, a fundamental property of telomeres (suppressing local DNA damage signaling so that the ends of linear chromosomes are not recognized as DNA double-strand breaks) results in oxidative telomeric damage being repaired much more slowly than elsewhere in the genome (Petersen, Saretzki et al. 1998), increasing the probability that single-strand damage gets converted into double-strand breaks and loss of telomeric sequences. Very long telomeres (too long to effectively count cell divisions) might allow large segments of the telomere to be lost without compromising cell division. Similarly, expressing telomerase would allow repair and elongation of any highly truncated telomeres. Maintaining telomeres short enough to limit the cellular proliferative capacity and function as a tumor suppressor mechanism might require an increased amount of resources invested in some oxidative protection mechanisms. This working hypothesis was examined by determining the sensitivity of 15 species to two types of oxidative stress, tert-Butyl hydroperoxide and sodium arsenite (Figure 2.5).

3.6. Telomere strategy and cellular oxidative stress

The relationship of sensitivity to telomere length as variable independent of size or lifespan was significant by maximum-likelihood in a phylogenetic generalized least

squares framework (PLGS) analysis for both agents (tert-Butyl hydroperoxide p=0.032, Fig. 2.5A; sodium arsenite p=0.017, Fig.2.5B). Many studies have found an association between lifespan or body weight and resistance to oxidative stress in cultured cells (e.g.(Kapahi, Boulton et al. 1999; Harper, Salmon et al. 2007)). This relationship also held with either lifespan or mass as independent variables (tert-Butyl hydroperoxide, PLGS log lifespan p=0.029, Fig. 2.5C, PLGS log body size p=0.016, data not shown; sodium arsenite, PLGS log lifespan p=0.0045, Fig. 2.5D; PLGS log body size p=0.0045, data not shown).

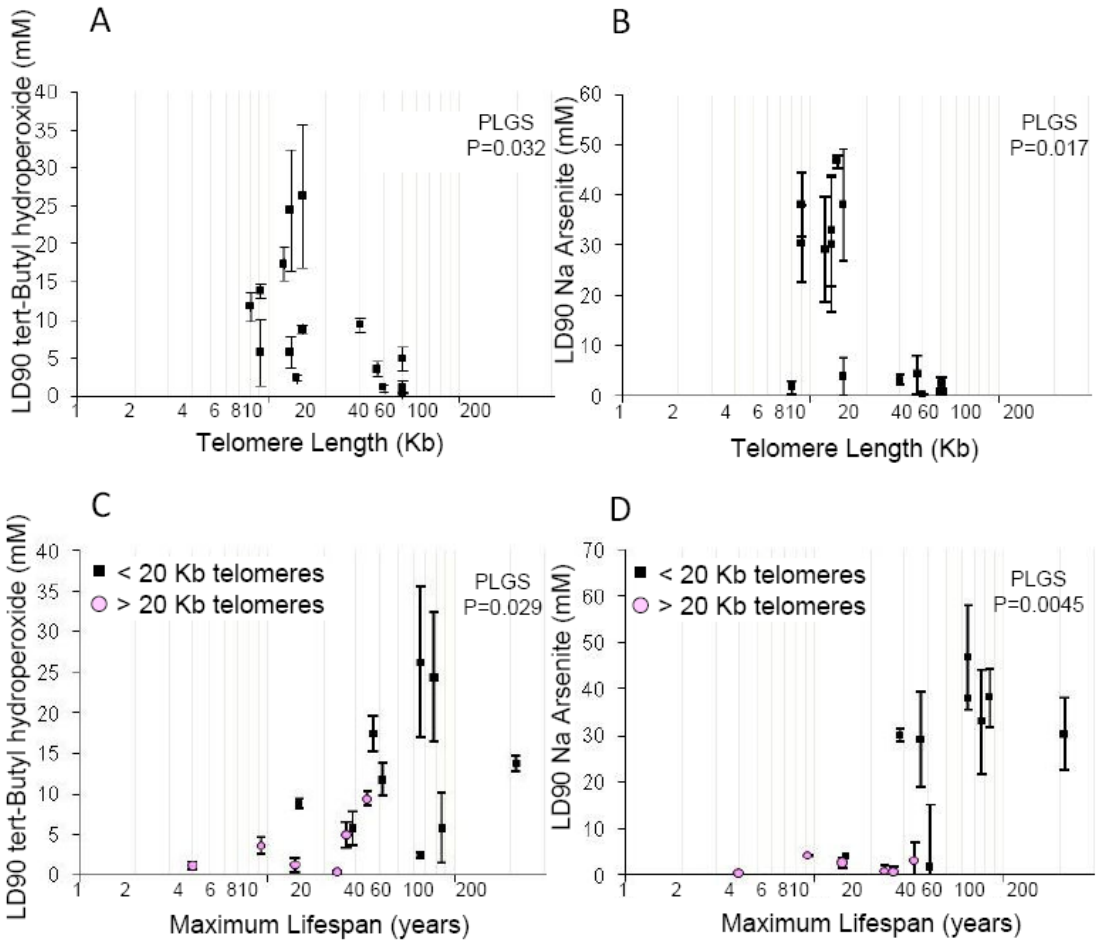


Figure 2.5. Resistance to tert-Butyl hydroperoxide and sodium arsenite .

The LD90 of a 4h treatment with different oxidative damage-inducing agents was examined for 12 different species. Data is shown for telomere length (A and B), maximum lifespan (C and D), Tert-Butyl hydroperoxide (A and C) and Sodium arsenite (B and D). The values for species with telomeres >20 Kb are shown in pink circles in C and D in order to permit the data to be more easily related to the plots of A and B. PLGS analysis demonstrates that resistance is significantly associated with telomere length independent of the effects on lifespan. Virtually identical patterns are observed if plotted

against body mass instead of lifespan (data not shown). Supplementary Table 3 gives the actual values for the specific species analyzed. +/- SEM of 2-6 titration curve.

The response to sodium arsenite is more dramatic than for tert-Butyl hydroperoxide. Species appeared to fall into two groups differing by 6-fold in their sensitivity without species in this limited analysis exhibiting intermediate values. The resistance to sodium arsenite was independent of the ability of the cells to grow well under standard culture conditions, since two species with short telomeres exhibiting stasis (grey whales and Malaysian tapirs, Supplemental Table S3) still exhibited the resistant phenotype. Although the mechanism for the different behavior of tert-Butyl hydroperoxide and sodium arsenite is unknown, it may reflect cellular compartmentalization, different propensities to damage lipid, protein, or DNA (Harper, Salmon et al. 2007), or the ability of arsenite to react with critical cysteines in some proteins (Flamigni, Marmiroli et al. 1989; Kapahi, Takahashi et al. 2000). Organic hydroperoxides such as tert-butyl hydroperoxide are cytotoxic and, although the exact mechanism of toxicity is unknown, it may involve peroxidation of cellular lipids, alkylation of cellular macromolecules, or alterations in cellular calcium homeostasis (Rush GF, Gorski JR et al. 1985). Arsenite is believed to exert its biological effects through reaction with exposed sulfhydryl groups, especially pairs of adjacent thiols. Sodium arsenite also has the ability to react with critical cysteines in some proteins, affecting for example the NF-kB signaling pathway (Kapahi, Takahashi et al. 2000).

Oxidative damage has long been hypothesized to be a major determinant of longevity(Harman 1956). The present result showing dependency on telomere length for both tert-Butyl hydroperoxide and sodium arsenite demonstrates that it will be important to consider telomere length as an independent variable in comparing the level of resistance to different oxidative stresses as a function of lifespan.

3.7. Telomere length and oxidative DNA damage

The next question addressed the relationship between the different telomere lenghts and more physiologic chronic oxidative DNA damage to the cells. In order to specifically assess oxidative DNA damage susceptibility/repair capacities of the cells versus overall damage to DNA the comet FLARE assay was used (2001; Parrinello, Samper et al. 2003). Although alkaline comet assay detects single strand DNA breaks, FLARE allows for the uncovering of specific damaged bases in the DNA, not only those that are alkali labile (Tice, Agurell et al. 2000; 2001; Olive and Banáth 2006). This technique allows the conversion of AP sites, DNA containing formamidopyrimidine moieties and 8 oxoguanine to alkali labile sites. The response to different O2 concentrations is presented in Fig. 2.6 and supplementary Table S4).

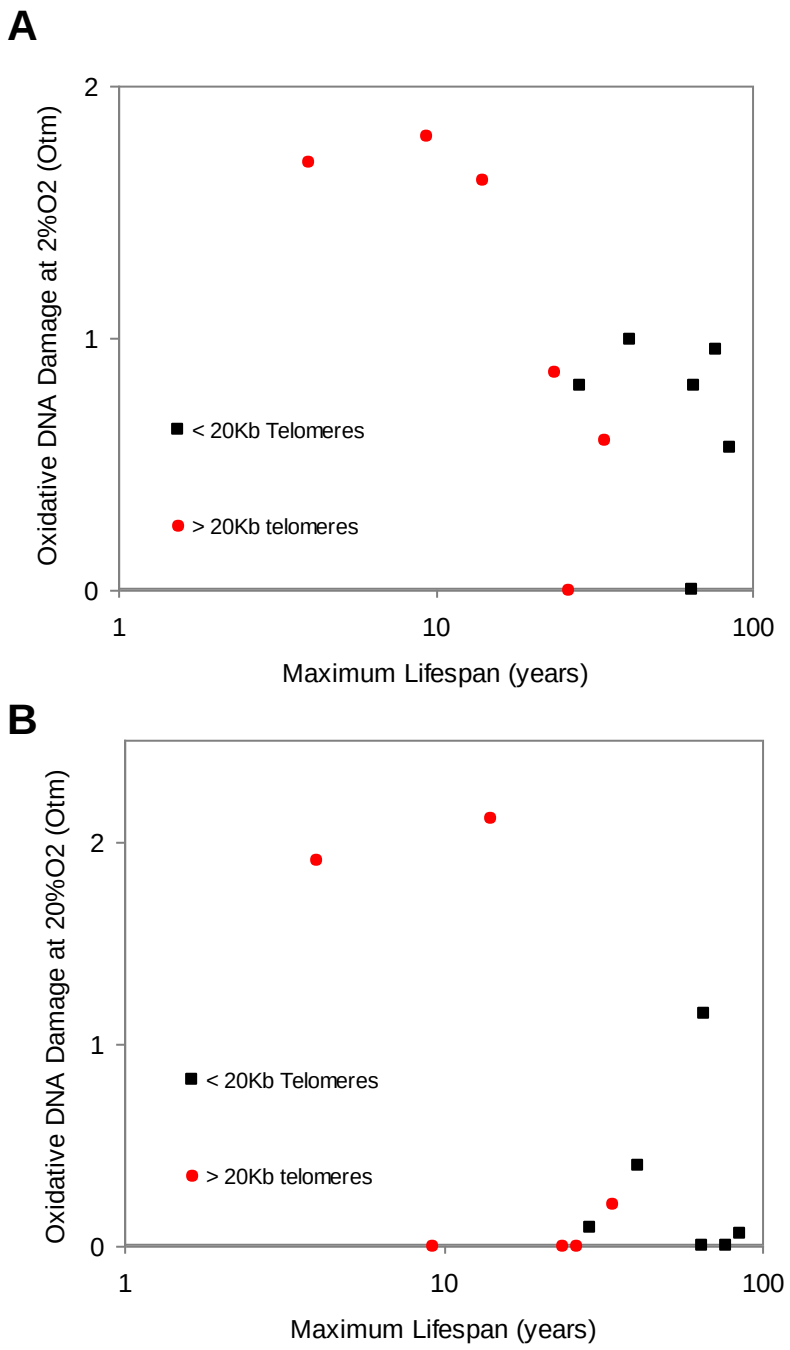


Figure 2.6. Oxidative DNA damage determined by comet (SCGE) FLARE negatively correlates with telomere length and longevity. Each point corresponds to the average of

2-4 SCGE-FLARE assays. Each assay is normalized by subtracting the average measurement of 100 nucleoids treated with Fpg minus the average of 100 buffer treated samples. A. Cells were grown at for at 2% O2 for 12 days. ($r_{LifespanOtm}$ =-0.55). B. Cells were grown at 20% O2 for 12 days. ($r_{LifespanOtm}$ =-0.38). Supplementary Table S4 gives the actual values for the specific species analyzed.

Oxidative DNA damage determined by comet (SCGE) FLARE negatively correlates with telomere length and longevity/body size. Some rodents such as mouse fibroblasts are clearly more susceptible to oxidative DNA damage compared to human fibroblasts (Fig. 2.6.). Species with long telomeres showed a small increase of the average oxidative damage versus the short telomere grouping (ratio of 1.6 in 2 % O2 and 2.5 in 20% O2). However, there was considerable overlap and a general weak correlation with maximum lifespan and telomere strategy. Higher resistance to damage was observed in the short telomere group, at both O2 concentrations. In the long telomere group, the higher resistance of the bat cells was not surprising since bats are know for their increased mechanisms of protection against ROS (Austad 1997; Brunet-Rossinni and Austad 2004). Also, several other species of this grouping showed a general resistance to oxidative DNA damage at 20% O2. The generalized observed lower damage at a higher O2 concentration is unexpected and might paradoxically reflect critically low levels of O2 after a few days of culture on the low oxygen containers (a gas mixture of 2% oxygen, 5% CO2 and 93% nitrogen was replaced every 3 days) (Wright and Shay 2006).

It is often very difficult to establish whether or not a species actually uses telomere shortening to count cell divisions (replicative aging). A large number of studies were required before one could conclude that the laboratory mouse did not use replicative aging (Sherr and DePinho 2000; Wright and Shay 2000). Although we believe that most of the species with telomeres much longer than ~20 kb do not use replicative aging, additional evidence is required to establish this conclusion. There is a clear tendency for species within variable orders larger than 1 kg to have short telomeres and repress telomerase (Fig. 2.3). Some of these (spotted hyena, domestic dog) may use replicative aging, since they growth-arrested in culture with short telomeres and were immortalized following the introduction of hTERT without needing to block p53 (Supplementary Fig. S1).

The hypothesis that there will be a direct relationship between telomere length and the number of available cell divisions among species that use replicative aging to count cell doublings is a reasonable initial hypothesis, but there are several observations that indicate this is unlikely to be true. One of the most important relates to the large difference in total potential cellular output as one varies the pattern of cell division by precursor cells. This can change from linear (one division generates one differentiated progeny and one stem cell, so that fifty division yields 50 differentiated cells) to exponential (where differentiated progeny are only produced at the final division, so 50 division would yield ~2^{50} cells). Cell division is the most important source of mutations, and one might anticipate that one would adjust the pattern of stem cell divisions in longer-lived animals in order to minimize mutations and tumor formation. Varied

patterns of stem cell division between species have not been adequately investigated, but there may be large differences depending on lifespan. In mice undergoing continuous 5-bromodeoxyuridine labeling to mark cell divisions, 50% of hematopoietic stem cells became labeled within six days (Cheshier 1999). When similar experiments were done in baboons, it took approximately 45-50 weeks for 50% labeling to occur (Mahmud 2001). This suggests that a much greater fraction of hematopoiesis is occurring from the "transient amplifying cell compartment" rather than the most primitive stem cells in baboons compared to mice, and that the rate of division of these primitive stem cells in baboons could be $1/50^{th}$ that in mice. This difference is roughly proportional to the difference in lifespan between these species, suggesting that the total number of stem cell divisions may be regulated to some optimal number over the lifespan. Similar arguments can apply to somatic stem cells throughout the body. As a consequence, we do not expect to find a direct correlation between small differences in telomere length and lifespan, but rather only the general correlation of whether or not telomeres are short enough to be used for replicative aging versus whether they are very long and used in a different fashion. I believe the strong PLGS relationship between telomere length as an independent variable and lifespan, where short telomeres are associated with longer lifespans, reflects this overall biology.

4. Conclusion

In summary, I have presented a general survey of telomere biology covering the mammalian radiation that shows that the ancestral mammalian phenotype had short telomeres and repressed telomerase, consistent with the hypothesis that the initial adaptation to homeothermy involved the adoption of replicative aging to compensate for the increased mutational load of elevated body temperatures. In addition, I find that telomere length inversely correlates with lifespan while telomerase expression correlates with mass. The role of replicative aging as a tumor suppression mechanism is well accepted, however its contribution to lifespan remains controversial. The demonstration that telomere length inversely correlates with lifespan provides support for the interpretation that replicative aging is one of many factors contributing to lifespan in a large number of species. The evidence that oxidative protection mechanisms are lower in species with long telomeres suggests one contribution to the evolutionary advantages of abandoning replicative aging in favor of long telomeres and not repressing telomerase. Comparative aging studies of stress resistance/oxidative protection will need to evaluate what changes are actually functions of lifespan versus more directly reflecting changes in telomere phenotype. These results now allow the role of telomeres in human cancer and aging to be put in the larger context of mammalian telomere biology.

5. Future Directions

In order to further elucidate the role of the telomeres as targets for free radical species it would be important to adapt and refine SCGE-FISH for telomeres of different animal species. It would also be important to distinguish between DNA damage susceptibility versus repair ability in these species and elucidate the specific telomeric DNA repair mechanisms involved. The recent technical improvement in the study of telomeric DNA overhangs (Yong Zhao, Shay et al. 2008) will allow the study of the eventual involvement of this telomeric component in these damage mechanisms and signaling, together with the further dissection of the involvement of the shelterin proteins in these processes.

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SUPPLEMENTARY TABLES

Supplementary Table S1. Species, Mass and Lifespan

Supplementary Table S1. Species, Mass and Lifespan

			Mass (kg)	Max long (yr)	Telomeres (Kb)	Telomerase (%H1299)	Stasis	Ars. Resist
Cetartiodactyla	Gray Whale	*Eschrichtius robustus*	28500	77	13	0	Y	Y
	Bowhead Whale	*Balaena mysticetus*	52,000	211	9	0	N	Y
	Bottlenosed Dolphin	*Tursiops truncatus*	190	55	17	0	N	
	Pygmy Hippopotamus	*Hexaprotodon liberiensis*	215	42.3	12	0	N	
	Giraffe	*Giraffa camelopardalis tippelskirchi*	800	40.5	10	0	N	
	Cow (literature)	*Bos taurus*	750	25	18	0	N	
	Sheep (literature)	*Ovis aries*	110	23	18	0	N	
	Indian Muntjac	*Muntiacus muntjak vaginalis*	21	18.8	14	0	N	
	Chinese Muntjac	*Muntiacus reevesi*	18	23.2	14	0	N	
	Pig (literature)	*Sus scrofa*	180	27	15	0	N	
	Dromedary Camel	*Camelus dromedarius*	495	28.4	13	0.3	N	Y
Perissodactyla	Southern White Rhinoceros	*Ceratotherium simum simum*	2175	45	10	0	N	
	Malayan Tapir	*Tapirus indicus*	275	36.5	12	0	Y	Y
	Horse	*Equus caballus*	250	45	14	0	N	
	Grevy's Zebra	*Equus grevyi*	340	31	12	0	N	
Carnivora	Spotted Hyena	*Crocuta crocuta*	63	41.1	8	0	N	N
	Indo-Chinese Tiger	*Panthera tigris corbetti*	162	26.3	50	10	Y	N
	Dog	*Canis familiaris*	20	24	15	0	N	
	Polar Bear	*Ursus maritimus*	475	43.8	12	0	Y	
	California Sea Lion	*Zalophus californianus*	180	35.7	5	0	Y	
	Red Panda	*Ailurus fulgens styani*	4.325	19	25	0	Y	
	Steppe Polecat	*Mustela putorius eversmannii*	1.35	9.1	40	0.3	Y	
Chiroptera	Rodrigues Flying Fox	*Pteropus rodricensis*	0.35	28	9	0	N	
	Big Brown Bat	*Eptesicus fuscus*	0.013	19	30	0.1	Y	N
	Little Brown Bat	*Myotis lucifugus*	0.008	34	30	10	N	
	Mexican Free-tailed Bat	*Tadarida brasiliensis*	0.01	12	26	10	N	
Eulipotyphla	African Pygmy Hedgehog	*Atelerix albiventris*	0.6	11.4	38	0.1	Y	
	Soricidae Shrew, Sand Diego, California	*probablly Notiosorex crafordii*	0.006	2	50	30	Y	
Rodentia	Mouse MEFS	*Mus musculus*	0.03	4	40	1	Y	N
	Norway Rat (literature)	*Rattus norvegicus*	0.35	4	40	1	Y	
	Sonoran Deer Mouse	*Peromyscus maniculatus sonoriensis*	0.021	8.3	9	0.1	Y	
	American Beaver	*Castor canadensis*	20.25	23.4	7	0	Y	
	Naked mole rat	*Heterocephalus glaber*	0.03	28	16	0.1	Y	
	Eastern Gray Squirrel	*Sciurus carolinensis*	0.553	23.6	50	100	Y	N
	Mountain Beaver	*Aplodontia rufa*	1.125	6	9	0	N	
Lagomorpha	European White Rabbit	*Oryctolagus cuniculus*	1.8	9	50	0	N	
	Black-tailed Jack Rabbit	*Lepus californicus*	4.175	11.8	25	0	N	
	Swamp Rabbit	*Sylvilagus aquaticus*	2.7	8	50	0	N	
	North american Pika	*Ochotona princeps*	0.1	7	22	10	N	
Scandentia	Large Tree Shrew	*Tupaia tana*	0.198	11.6	15	0	Y	
Primates	Ring-tailed Lemur	*Lemur catta*	2.555	37.3	19	0	N	
	Black-handed Spider Monkey	*Ateles geoffroyi*	7.268	47.1	7	0	N	
	Squirrel Monkey	*Saimiri sciureus*	0.925	30.2	9	0	N	
	Rhesus Monkey	*Macaca mulattta*	8.235	40	16	0	N	
	Orangutan	*Pongo pygmaeus*	64.475	59	10	0	N	
	Pigmy Chimpanzee	*Pan paniscus*	39.925	55	10	0	N	
	Human BJ / IMR90 / SW26/ SW39	*Homo sapiens*	51	85	9	0	N	Y
Xenarthra	Two-toed Sloth	*Choloepus hoffmanni*	6.25	35	17	0	N	
	Giant Anteater	*Myrmecophaga t. tridactyla*	28.5	31	12	0	N	
	Lesser Hairy Armadillo	*Chaetophractus vellerosus*	0.84	16.2	13	0	N	
Afrosoricida	Large Madagascar Hedgehog Tenrec	*Setifer setosus*	0.225	14.1	50	100	Y	N
Macroscelidea	Short-eared Elephant Shrew	*Macroscelides proboscideus*	0.04	8.7	37	0.3	Y	
	Long-eared Elephant shrew	*Elephantulus rufescens*	0.058	9.3	37	1	Y	N
Hyracoidea	Rock Hyrax	*Procavia capensis*	3.6	14.8	15	0	N	N
Proboscidea	Indian Elephant	*Elephas maximus*	3178	65.5	15	0	N	Y
	African Elephant	*Loxodonta africana*	4800	65	14	0	N	Y
Diprotodontia	Red kangaroo	*Macropus rufus*	55	25	I	0	N	
	Koala	*Phascolarctos cinereus*	9.3	22.1	I	0	Y	
	Wombat	*Lasiorhinus latifrons*	25.5	30	I	0	Y	
Didelphimorphia	Virginia Opossum	*Didelphis virginiana*	3	6.6	35	0.3	Y	
Monotremata	Short-beaked Echidna	*Tachyglossus aculeatus*	4.25	49.5	I	0	N	

Supplementary Table S2. PLGS analysis of individual non-placental mammalian orders[1]

Order	Estimated Ancestral Telomere Length (Kb)	Probability Telomerase Repressed	Probability Telomerase Expressed
Cetartiodactyla	15.37450956	1	0
Perissodactyla	13.68474106	1	0
Carnivora	16.840399	1	0
Chiroptera	17.95100871	1	0
Eulipotyphla	22.95807157	0.069227	0.930773
Rodentia	17.85377852	1	0
Lagomorpha	23.44728276	1	0
Primates	16.91966772	1	0
Xenarthra	16.03829563	1	0
Macroscelidea	34.50944277	0.007023	0.992977
Proboscidea	14.98941078	1	0

[1]Data were analyzed using phylogeny within the phylogenetic least-squares framework (PLGS) (Pagel 1999; Freckleton, Harvey et al. 2002; Pagel, Meade et al. 2004) with a model of evolution estimated across the entire tree to estimate ancestral states (Organ, Janes et al. 2009)**.** Orders represented by only one species were not included.

Supplementary Table S3. Species analyzed for resistance to oxidative stressors

Length	Telomerase	Stasis	Species	Max. Lifespan	Size (Kg)	tert-Butyl hydroperoxide LD90 (mM) ± SE	Na arsenite LD90 (mM) ± SE	
9	0	N	Bowhead Whale	211	52000	13.8 ± 0.9	30.4 ± 7.7	Telomeres<20Kb
13	0	Y	Gray Whale	77	28,500	24.4 ± 8.0	32.9 ± 11.2	
12	0	Y	Malasian Tapir	36.5	275	17.4 ± 2.2	29.2 ± 10.4	
9	0	N	Human BJ fibroblasts	85	51	5.8 ± 4.4	38.2 ± 6.4	
15	0	N	Indian Elephant	65.5	3178	26.3 ± 9.3	38.1 ± 11.2	
14	0	N	African Elephant	65	4800	2.5 ± 0.3	46.7 ± 1.3	
13	0.3	N	Camel	28.4	495	5.8 ± 2.1	30.1 ± 13.3	
8	0	N	Hyena	41.1	63	11.8 ± 2.0	1.8 ± 1.3	
15	0	N	Rock Hyrax	14.8	3.6	8.8 ± 0.6	3.9 ± 3.7	
50	10	Y	Tiger	26.3	162	5.0 ± 1.6	0.5 ± 0.2	Telomeres>20Kb
37	1	Y	L.E. Elephant Shrew	9.3	0.058	3.6 ± 1.0	4.3 ± 3.8	
30	10	N	Little Brown Bat	34	0.008	9.4 ± 0.9	3.2 ± 1.1	
50	100	Y	Madagascar Hedgehog Tenrec	14.1	0.428	1.2 ± 0.9	2.6 ± 1.3	
50	100	Y	Squirrel	23.6	0.553	0.3 ± 0.2	1.0 ± 0.2	
40	1	Y	Mouse embryo fibroblasts	4	0.03	1.1 ± 0.5	0.4 ± 0.1	

Supplementary Table S4. Species analyzed for resistance to different oxygen concentrations.

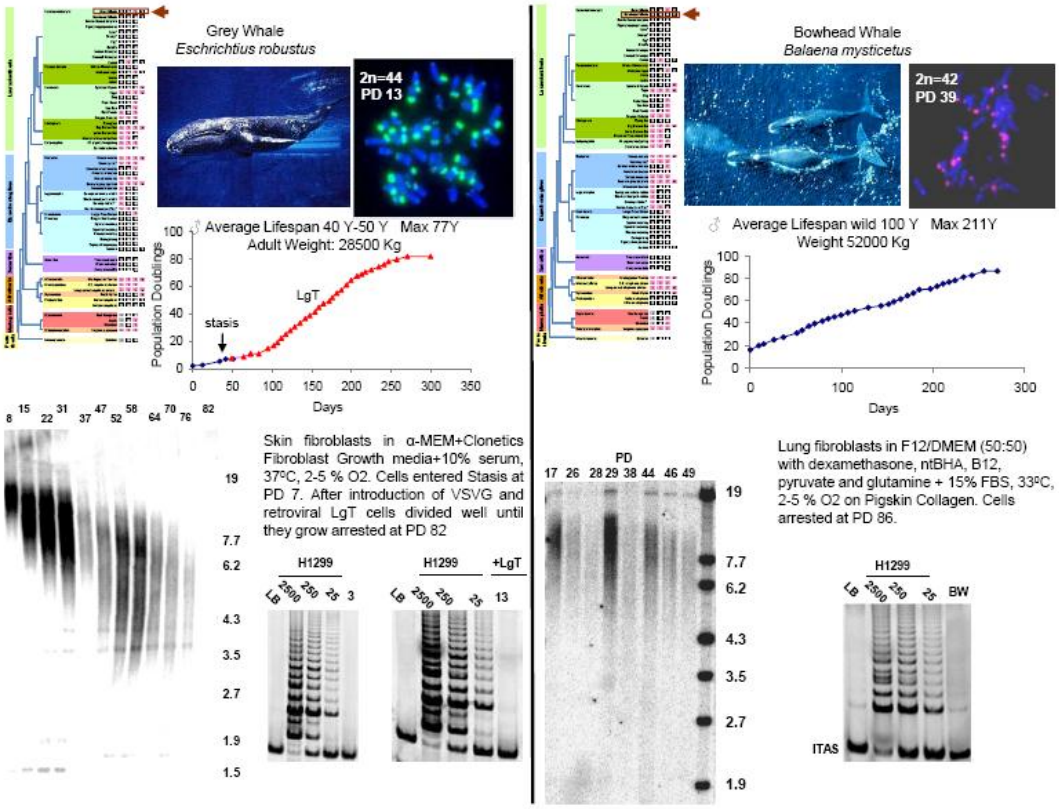
Length	Telomerase	Stasis	Species	Max. Lifespan	Size (Kg)	DNA Damage at 2%O2 (Otm) ± SE	DNA Damage at 20%O2 (Otm) ± SE	
S	N	Y	Gray Whale	77	28,500	0.95 ± 1.34	0 ± 0	
S	N	N	Human BJ fibroblasts	85	51	0.56 ± 0.33	0.06 ± 0.08	< 20 Kb Telomeres
S	N	N	Indian Elephant	65.5	3178	0.81 ± 0.33	1.15 ± 0.63	
S	N	N	African Elephant	65	4800	0 ± 0	0 ± 0	
S	Y	N	Camel	28.4	495	0.81 ± 0	0.08 ± 0	
S	N	N	Hyena	41.1	63	0.99 ± 0.99	0.39 ± 0.39	
L	Y	Y	Tiger	26.3	162	0 ± 0	0 ± 0	
L	Y	Y	L.E. Elephant Shrew	9.3	0.058	1.80 ± 1.74	0 ± 0	> 20Kb Telomeres
L	Y	N	Little Brown Bat	34	0.008	0.60 ± 0.37	0.21 ± 0.23	
L	Y	Y	Greater Hedgehog Tenrec	14.1	0.428	1.63 ± 1.26	2.11 ± 2.11	
L	Y	Y	Squirrel	23.6	0.553	0.87 ± 0.87	0 ± 0	
L	Y	Y	Mouse embryo fibroblasts	4	0.03	1.70 ± 2.41	1.90 ± 2.36	

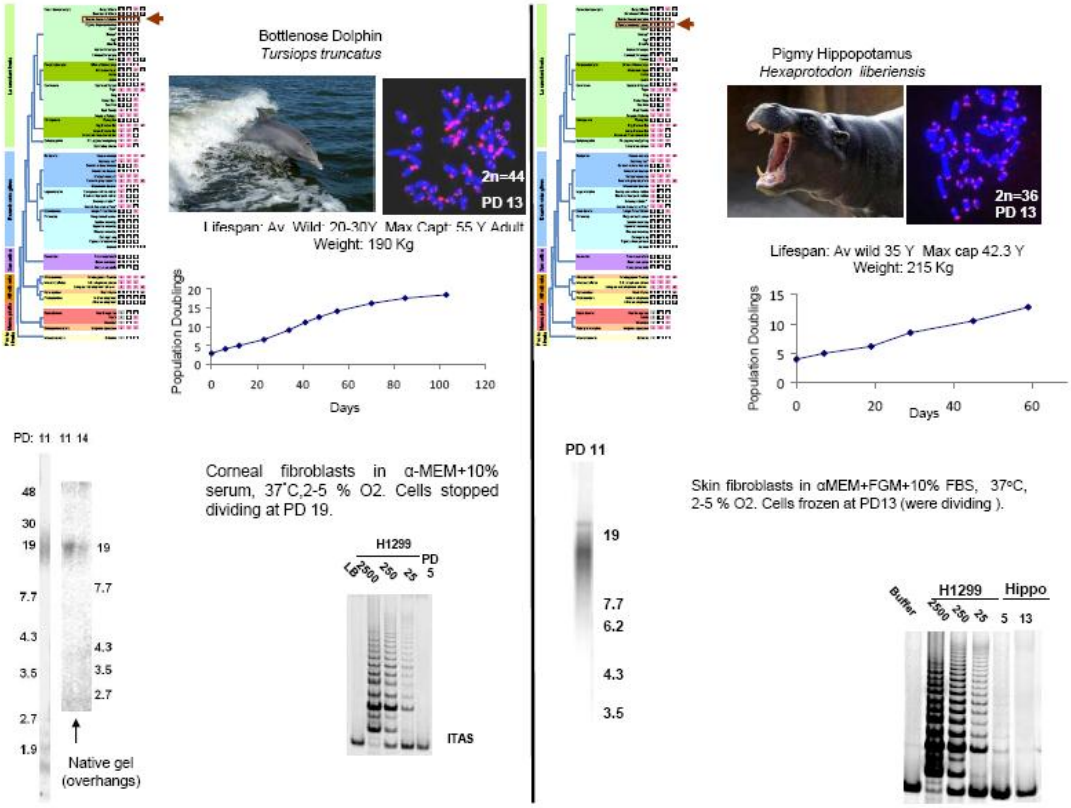
SUPPLEMENTARY FIGURES

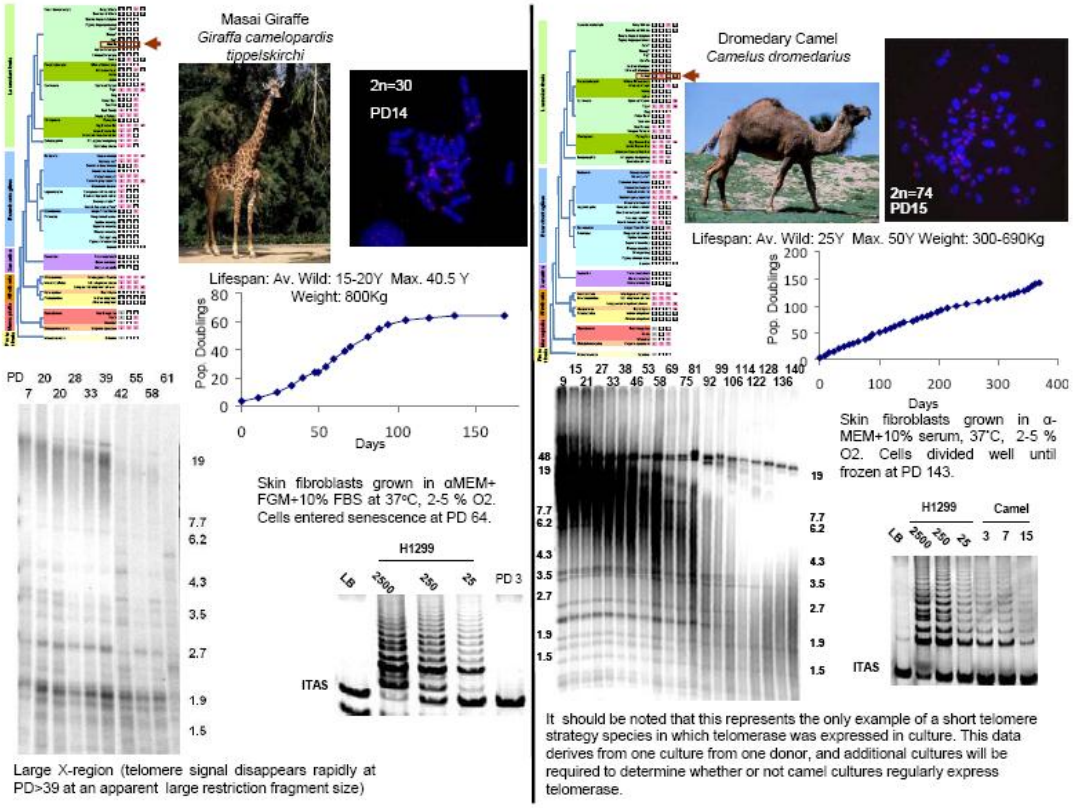
Supplementary Figure S1. Data on growth, telomere length and telomerase expression for 43 species.

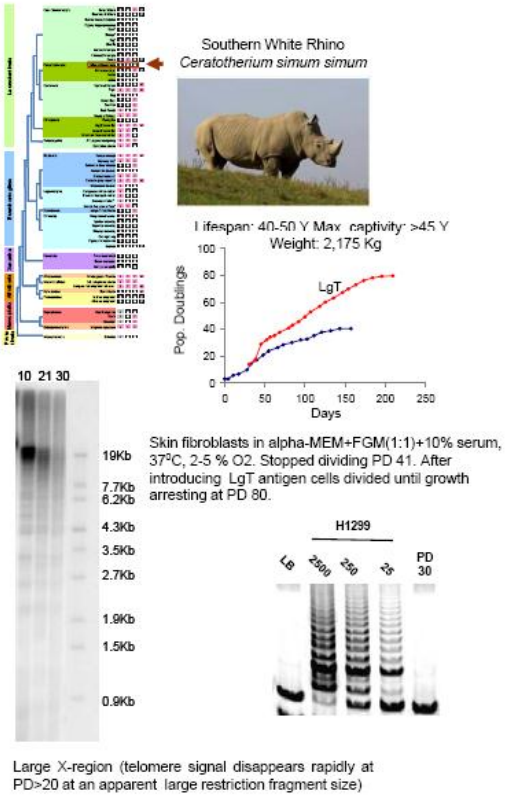
Growth curves were determined at each subcultivation and are expressed as cumulative population doublings versus time. Telomere Restriction Fragment Analysis is shown as a function of population doublings in culture (PD). Telomerase TRAP assays are shown in comparison with a reference serial 10-fold dilution of H1299 Lung Adenocarcinoma cells. The band at the bottom of the gel is the internal TRAP assay standard (ITAS) used to establish linearity (if the telomerase extension products are too great they inhibit amplification of the ITAS PCR standard). Data on weight, mean and maximum lifespan are from AnAge, the Animal Ageing & longevity Database (http://genomics.senescence.info/species/).

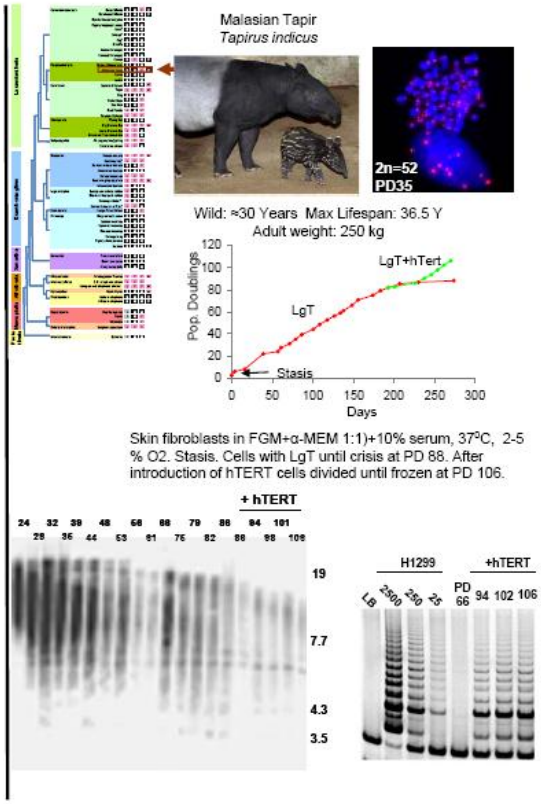
Photo Credits

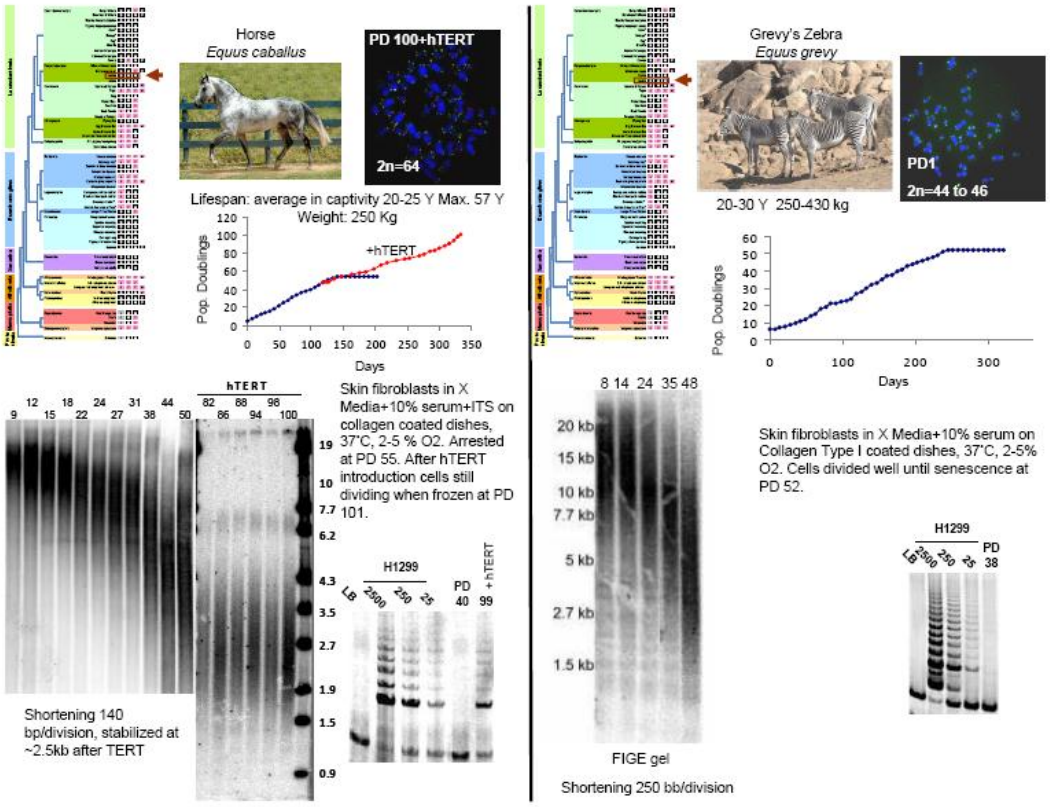

Grey Whale
Eschrichtius robustus
2n=44
PD 13
♂ Average Lifespan 40 Y-50 Y Max 77Y
Adult Weight: 28500 Kg
Population Doublings
stasis
LgT
Days
Skin fibroblasts in α-MEM+Clonetics Fibroblast Growth media+10% serum, 37ºC, 2-5 % O2. Cells entered Stasis at PD 7. After introduction of VSVG and retroviral LgT cells divided well until they grow arrested at PD 82
H1299
+LgT
LB
2500
250
25
3
13
Bowhead Whale
Balaena mysticetus
2n=42
PD 39
♂ Average Lifespan wild 100 Y Max 211Y
Weight 52000 Kg
Population Doublings
Days
PD
Lung fibroblasts in F12/DMEM (50:50) with dexamethasone, ntBHA, B12, pyruvate and glutamine + 15% FBS, 33ºC, 2-5 % O2 on Pigskin Collagen. Cells arrested at PD 86.
BW
ITAS

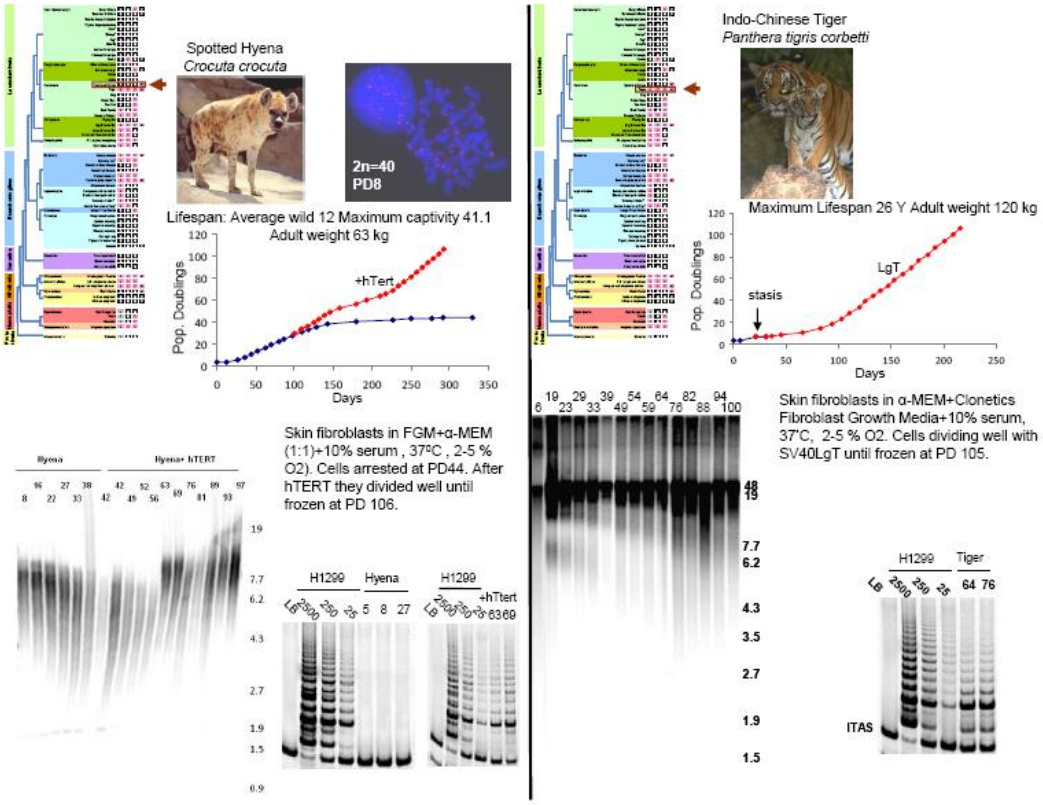

Bottlenose Dolphin
Tursiops truncatus
2n=44
PD 13
Lifespan: Av. Wild: 20-30Y. Max Capt: 55 Y Adult
Weight: 190 Kg
Population Doublings
Days
Pigmy Hippopotamus
Hexaprotodon liberiensis
2n=36
PD 13
Lifespan: Av wild 35 Y Max cap 42.3 Y
Weight: 215 Kg
Population Doublings
Days
PD: 11 11 14
48
30
19
7.7
4.3
3.5
2.7
1.9
19
7.7
4.3
3.5
2.7
Native gel
(overhangs)
Corneal fibroblasts in α-MEM+10% serum, 37˚C,2-5 % O2. Cells stopped dividing at PD 19.
H1299
LB
2500
250
25
PD 5
ITAS
PD 11
19
7.7
6.2
4.3
3.5
Skin fibroblasts in αMEM+FGM+10% FBS, 37°C, 2-5 % O2. Cells frozen at PD13 (were dividing).
Buffer
H1299
2500
250
25
Hippo
5
13

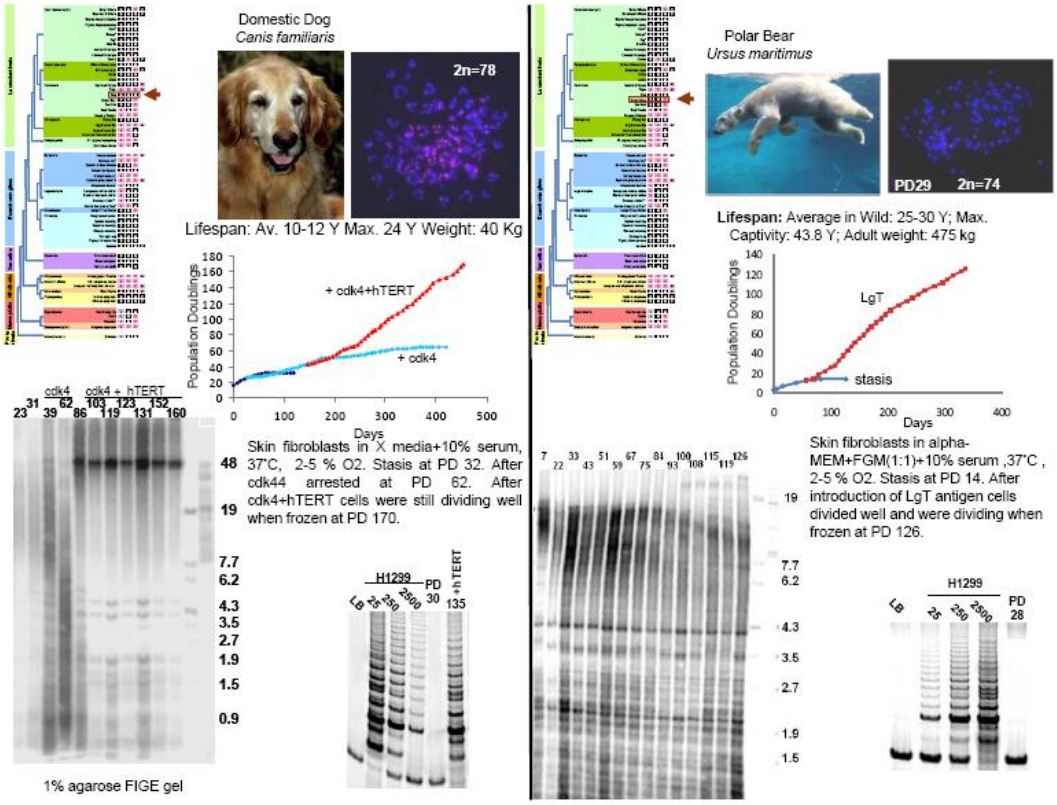

Masai Giraffe
Giraffa camelopardis tippelskirchi
2n=30
PD14
Lifespan: Av. Wild: 15-20Y Max. 40.5 Y
Weight: 800Kg
Pop. Doublings
Days
Skin fibroblasts grown in αMEM+ FGM+10% FBS at 37ºC, 2-5 % O2. Cells entered senescence at PD 64.
H1299
LB
2500
250
25
PD 3
ITAS
PD 20 28 39 55 61
7 20 33 42 58
Large X-region (telomere signal disappears rapidly at PD>39 at an apparent large restriction fragment size)
Dromedary Camel
Camelus dromedarius
2n=74
PD15
Lifespan: Av. Wild: 25Y Max. 50Y Weight: 300-690Kg
Pop. Doublings
Days
9 21 33 46 58 75 92 106 122 136
15 27 38 53 69 81 99 114 128 140
Skin fibroblasts grown in α-MEM+10% serum, 37°C, 2-5 % O2. Cells divided well until frozen at PD 143.
H1299
Camel
LB
2500
250
25
3 7 15
ITAS
It should be noted that this represents the only example of a short telomere strategy species in which telomerase was expressed in culture. This data derives from one culture from one donor, and additional cultures will be required to determine whether or not camel cultures regularly express telomerase.

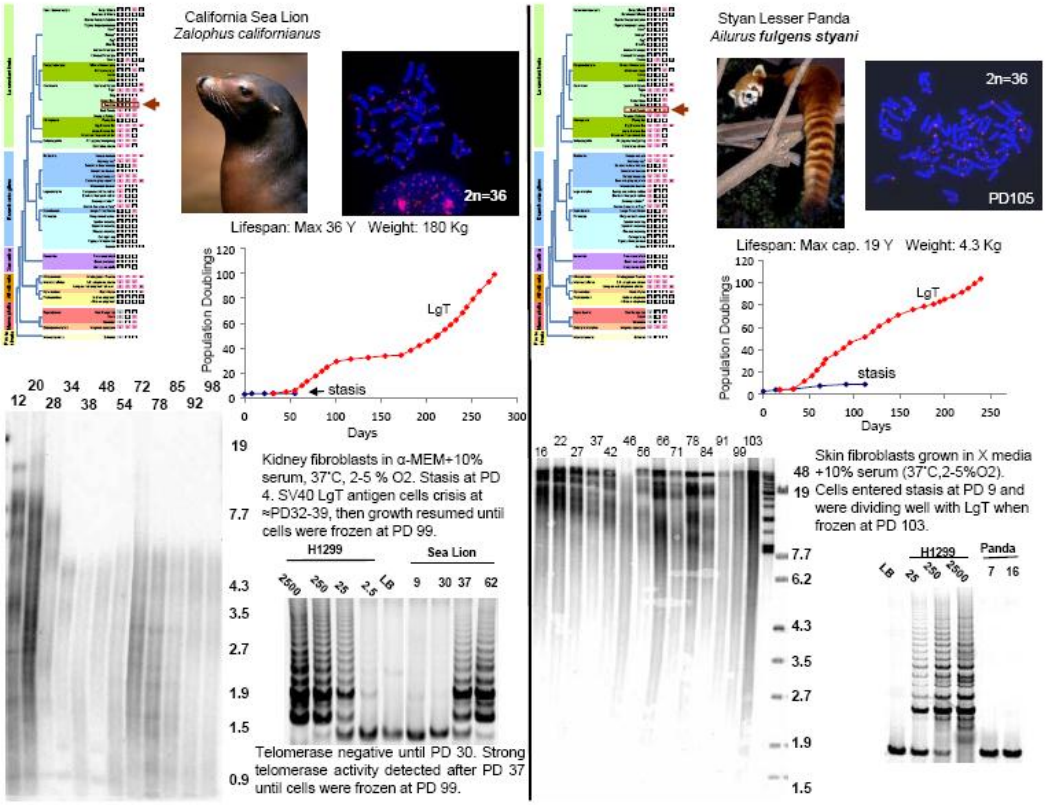

Southern White Rhino
Ceratotherium simum simum
Lifespan: 40-50 Y Max. captivity: >45 Y
Weight: 2,175 Kg
Pop. Doublings
LgT
Days
10 21 30
19Kb
7.7Kb
6.2Kb
4.3Kb
3.5Kb
2.7Kb
1.9Kb
1.5Kb
0.9Kb
H1299
LB
2500
250
25
PD 30
Skin fibroblasts in alpha-MEM+FGM(1:1)+10% serum, 37°C, 2-5 % O2. Stopped dividing PD 41. After introducing LgT antigen cells divided until growth arresting at PD 80.
Large X-region (telomere signal disappears rapidly at PD>20 at an apparent large restriction fragment size)

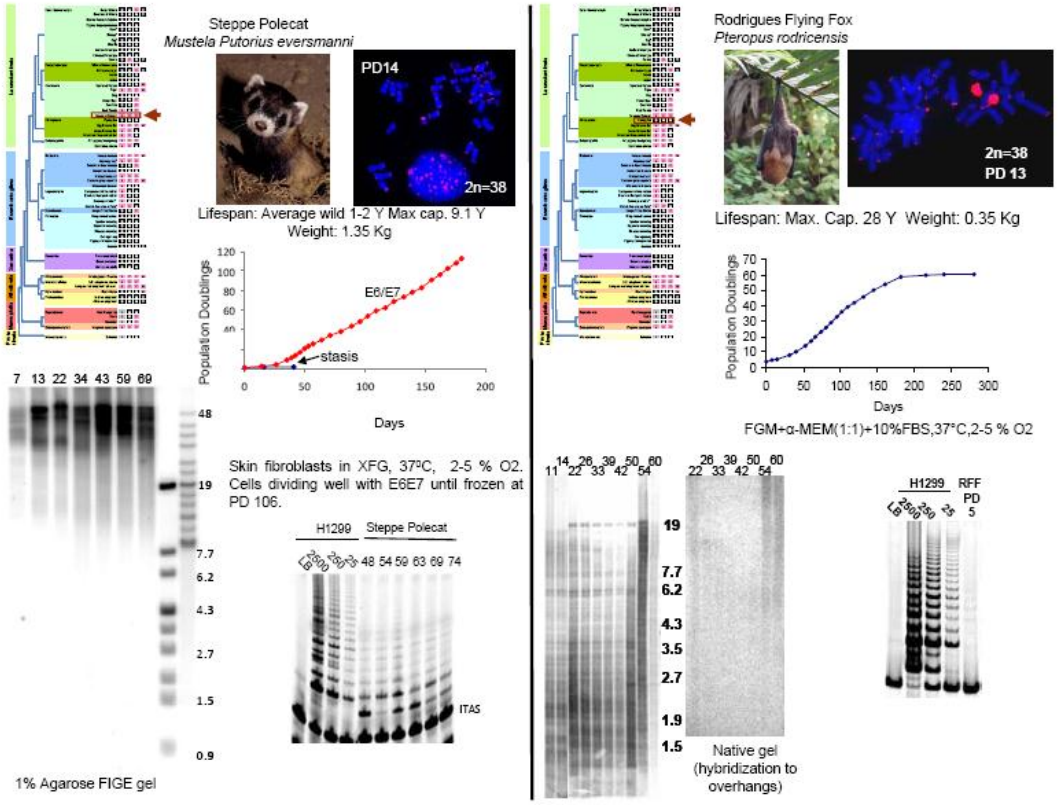

Malasian Tapir
Tapirus indicus
2n=52
PD35
Wild: ≈30 Years Max Lifespan: 36.5 Y
Adult weight: 250 kg
Pop. Doublings
LgT+hTert
LgT
Stasis
Days
Skin fibroblasts in FGM+α-MEM 1:1)+10% serum, 37°C, 2-5 % O2. Stasis. Cells with LgT until crisis at PD 88. After introduction of hTERT cells divided until frozen at PD 106.
+ hTERT
24 32 39 48 68 88 79 86 94 101
28 36 44 53 61 76 82 88 98 106
19
7.7
4.3
3.5
H1299
+hTERT
LB
2500
250
25
PD 66
94
102
106

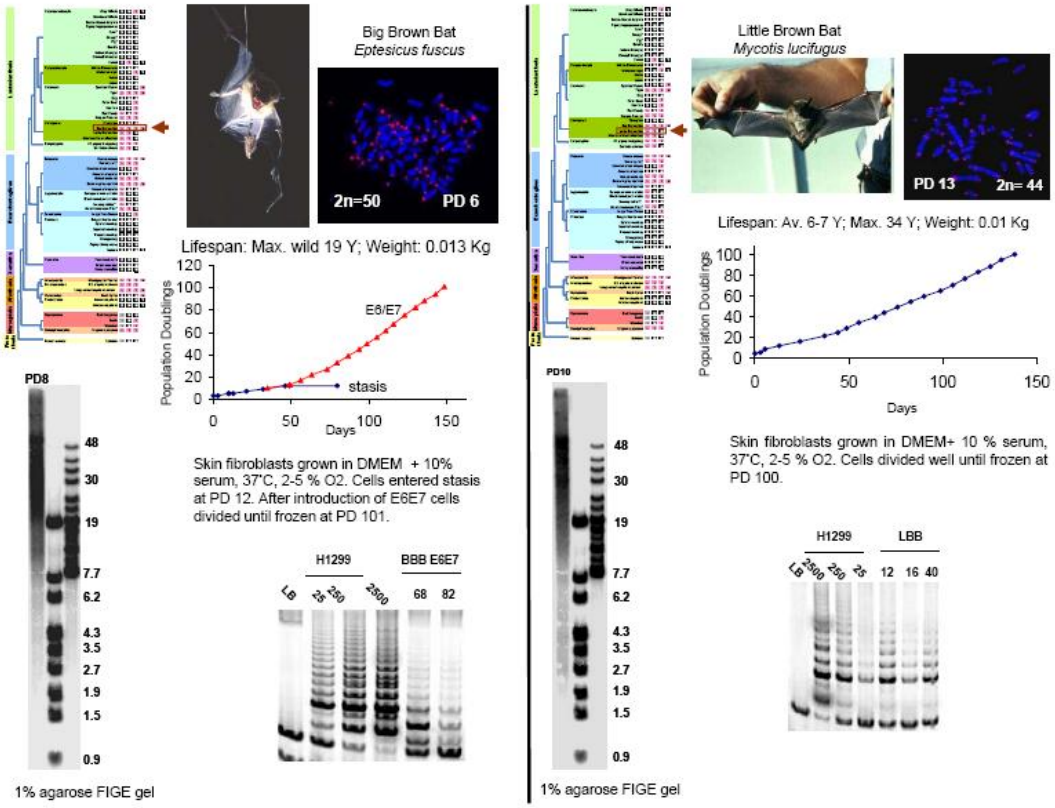

Horse
Equus caballus
PD 100+hTERT
2n=64
Lifespan: average in captivity 20-25 Y Max. 57 Y
Weight: 250 Kg
+hTERT
Pop. Doublings
Days
hTERT
Skin fibroblasts in X Media+10% serum+ITS on collagen coated dishes, 37°C, 2-5 % O2. Arrested at PD 55. After hTERT introduction cells still dividing when frozen at PD 101.
H1299
LB
2500
250
25
PD 40
99
+ hTERT
Shortening 140 bp/division, stabilized at ~2.5kb after TERT
Grevy's Zebra
Equus grevy
PD1
2n=44 to 46
20-30 Y 250-430 kg
Pop. Doublings
Days
Skin fibroblasts in X Media+10% serum on Collagen Type I coated dishes, 37°C, 2-5% O2. Cells divided well until senescence at PD 52.
8 14 24 35 48
20 kb
15 kb
10 kb
7.7 kb
5 kb
2.7 kb
1.5 kb
FIGE gel
Shortening 250 bb/division
H1299
LB
2500
250
25
PD 38

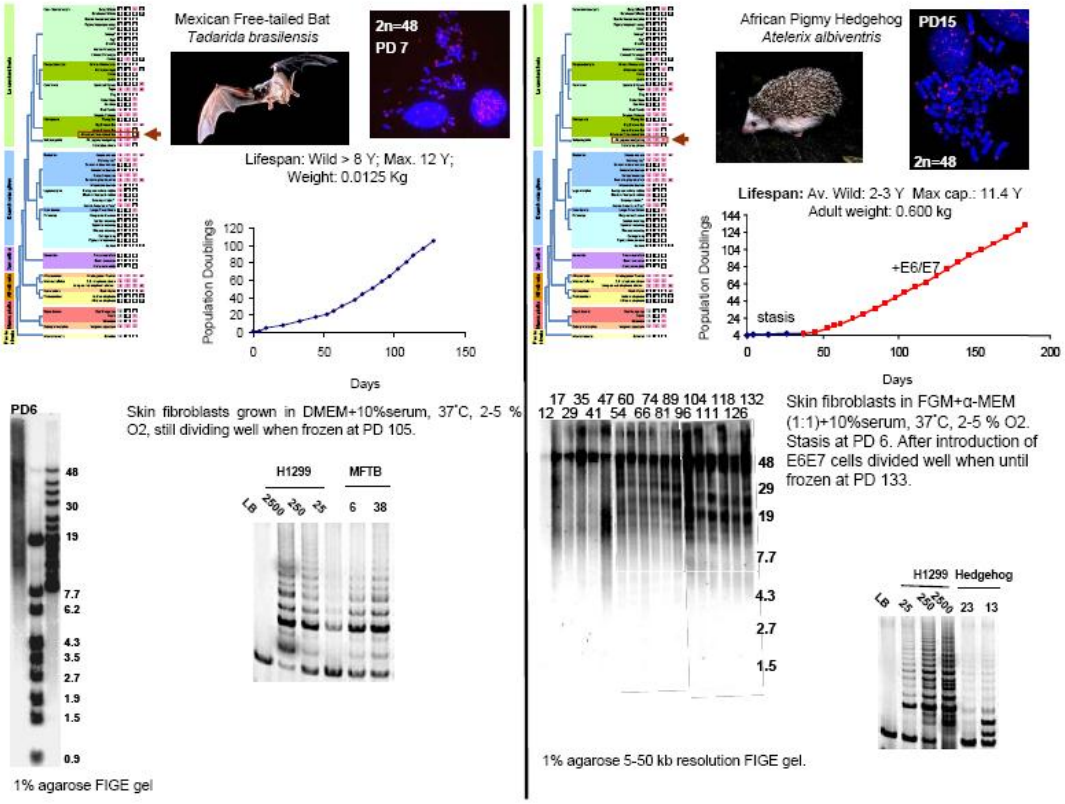

Spotted Hyena
Crocuta crocuta
2n=40
PD8
Lifespan: Average wild 12 Maximum captivity 41.1
Adult weight 63 kg
Pop. Doublings
+hTert
Days
Skin fibroblasts in FGM+α-MEM (1:1)+10% serum , 37ºC , 2-5 % O2). Cells arrested at PD44. After hTERT they divided well until frozen at PD 106.
Hyena
Hyena+ hTERT
H1299
Hyena
H1299
+hTert
Indo-Chinese Tiger
Panthera tigris corbetti
Maximum Lifespan 26 Y Adult weight 120 kg
stasis
LgT
Pop. Doublings
Days
Skin fibroblasts in α-MEM+Clonetics Fibroblast Growth Media+10% serum, 37°C, 2-5 % O2. Cells dividing well with SV40LgT until frozen at PD 105.
H1299
Tiger
ITAS

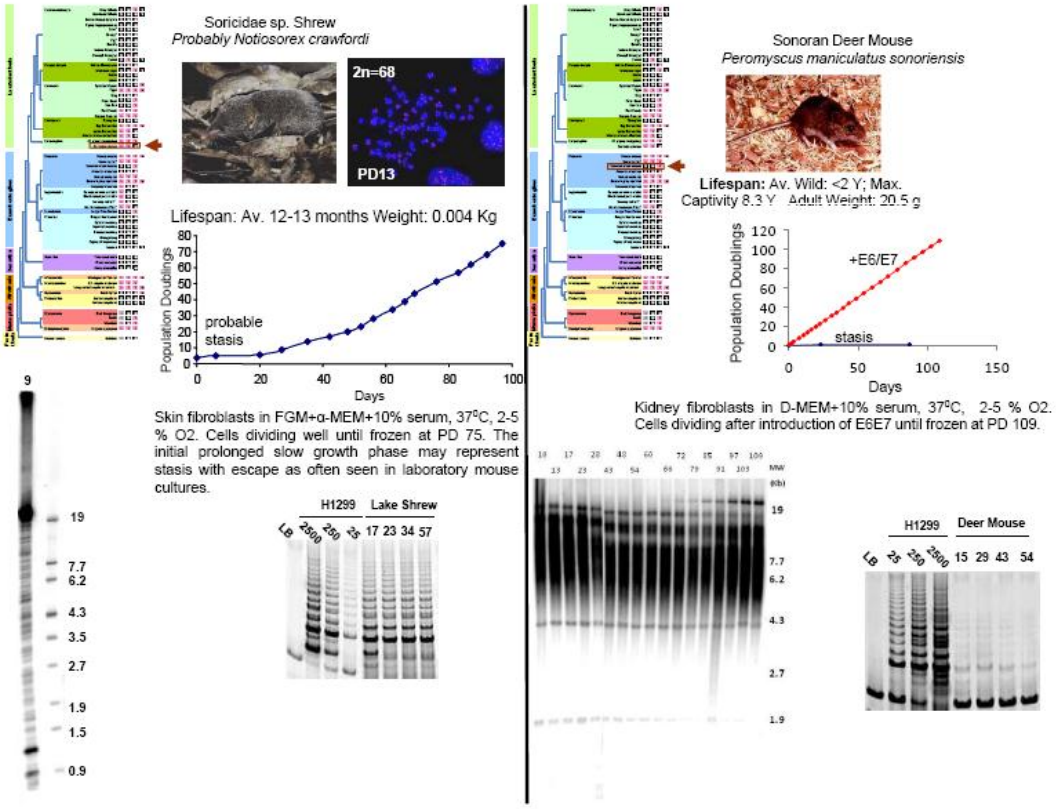

Domestic Dog
Canis familiaris
2n=78
Lifespan: Av. 10-12 Y Max. 24 Y Weight: 40 Kg
Population Doublings
+ cdk4+hTERT
+ cdk4
Days
Skin fibroblasts in X media+10% serum, 37°C, 2-5 % O2. Stasis at PD 32. After cdk44 arrested at PD 62. After cdk4+hTERT cells were still dividing well when frozen at PD 170.
cdk4
cdk4 + hTERT
1% agarose FIGE gel
H1299
PD
+hTERT
LB
Polar Bear
Ursus maritimus
PD29
2n=74
Lifespan: Average in Wild: 25-30 Y; Max. Captivity: 43.8 Y; Adult weight: 475 kg
LgT
stasis
Skin fibroblasts in alpha-MEM+FGM(1:1)+10% serum ,37°C , 2-5 % O2. Stasis at PD 14. After introduction of LgT antigen cells divided well and were dividing when frozen at PD 126.

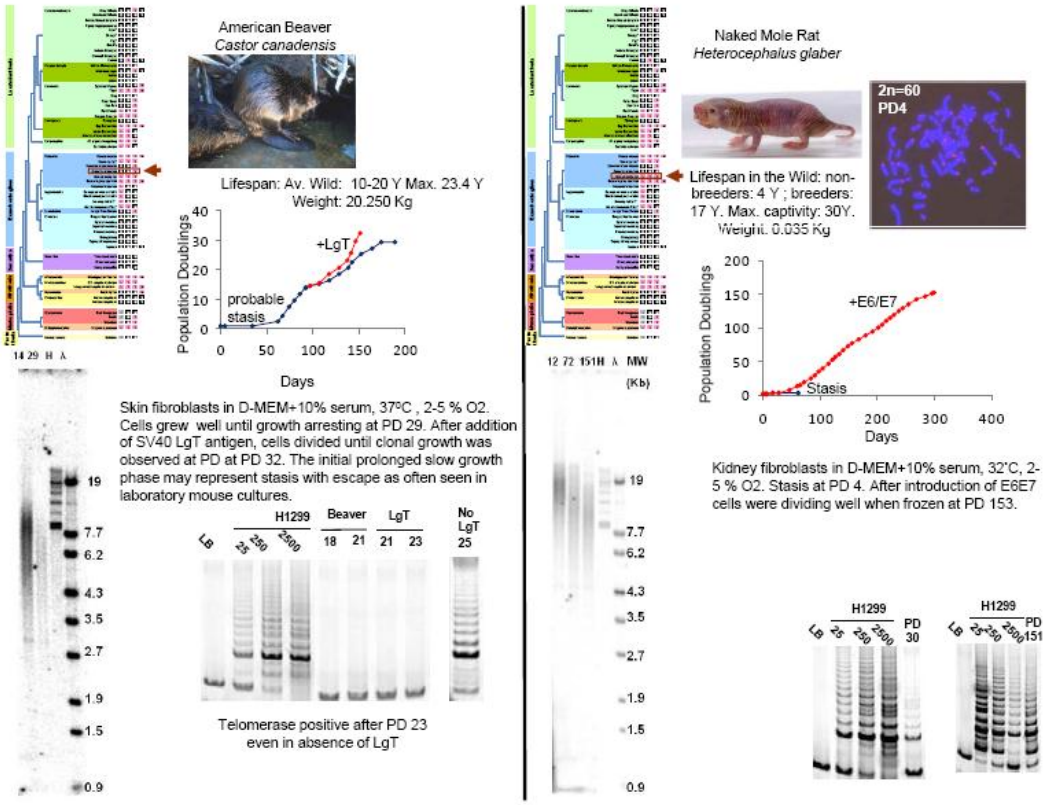

California Sea Lion
Zalophus californianus
2n=36
Lifespan: Max 36 Y Weight: 180 Kg
Population Doublings
LgT
stasis
Days
20 34 48 72 85 98
12 28 38 54 78 92
19
7.7
Kidney fibroblasts in α-MEM+10% serum, 37°C, 2-5 % O2. Stasis at PD 4. SV40 LgT antigen cells crisis at ≈PD32-39, then growth resumed until cells were frozen at PD 99.
H1299
Sea Lion
2500 250 25 2.5 LB 9 30 37 62
4.3
3.5
2.7
1.9
1.5
0.9
Telomerase negative until PD 30. Strong telomerase activity detected after PD 37 until cells were frozen at PD 99.
Styan Lesser Panda
Ailurus fulgens styani
2n=36
PD105
Lifespan: Max cap. 19 Y Weight: 4.3 Kg
Population Doublings
LgT
stasis
Days
16 22 27 37 42 46 56 66 71 78 84 91 99 103
48
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
Skin fibroblasts grown in X media +10% serum (37°C,2-5%O2). Cells entered stasis at PD 9 and were dividing well with LgT when frozen at PD 103.
H1299
Panda
LB 25 250 2500 7 16

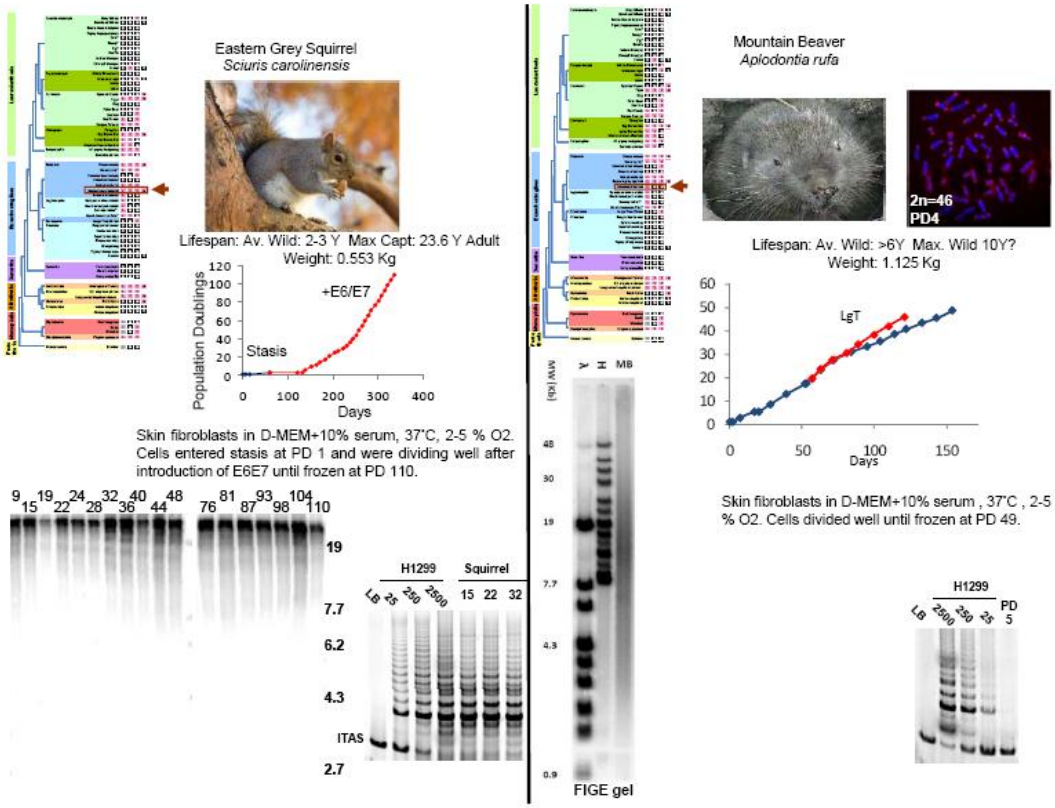

Steppe Polecat
Mustela Putorius eversmanni
PD14
2n=38
Lifespan: Average wild 1-2 Y Max cap. 9.1 Y
Weight: 1.35 Kg
Population Doublings
E6/E7
stasis
Days
7 13 22 34 43 59 69
48
19
7.7
6.2
4.3
2.7
1.5
0.9
1% Agarose FIGE gel
Skin fibroblasts in XFG, 37ºC, 2-5 % O2. Cells dividing well with E6E7 until frozen at PD 106.
H1299
Steppe Polecat
LB 25 250 3 48 54 59 63 69 74
ITAS
Rodrigues Flying Fox
Pteropus rodricensis
2n=38
PD 13
Lifespan: Max. Cap. 28 Y Weight: 0.35 Kg
Population Doublings
Days
FGM+α-MEM(1:1)+10%FBS,37°C,2-5 % O2
11 14 22 26 33 39 42 50 54 60
22 26 33 39 42 50 54 60
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
Native gel
(hybridization to overhangs)
H1299
RFF
LB 2500 250 25
PD 5

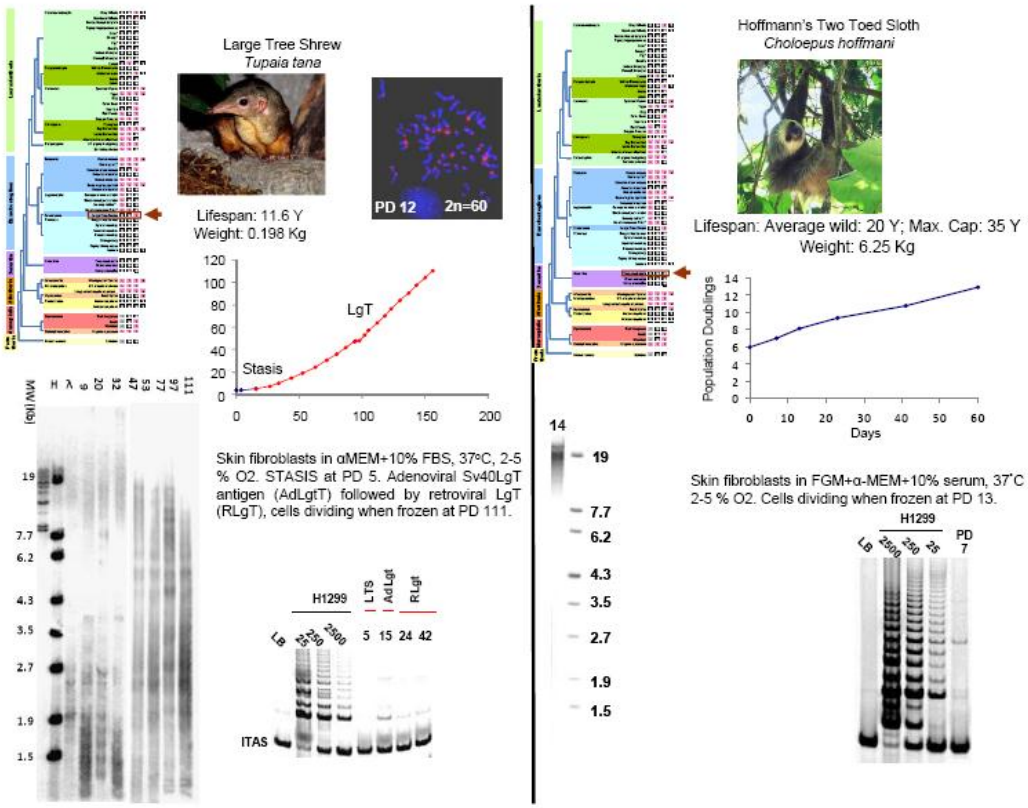

Big Brown Bat
Eptesicus fuscus
2n=50
PD 6
Lifespan: Max. wild 19 Y; Weight: 0.013 Kg
Population Doublings
E6/E7
stasis
Days
Skin fibroblasts grown in DMEM + 10% serum, 37°C, 2-5 % O2. Cells entered stasis at PD 12. After introduction of E6E7 cells divided until frozen at PD 101.
H1299
BBB E6E7
LB
25
250
2500
68
82
PD8
48
30
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
0.9
1% agarose FIGE gel
Little Brown Bat
Mycotis lucifugus
PD 13
2n= 44
Lifespan: Av. 6-7 Y; Max. 34 Y; Weight: 0.01 Kg
Population Doublings
Days
Skin fibroblasts grown in DMEM+ 10 % serum, 37°C, 2-5 % O2. Cells divided well until frozen at PD 100.
H1299
LBB
LB
2500
250
25
12
16
40
PD10
48
30
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
0.9
1% agarose FIGE gel

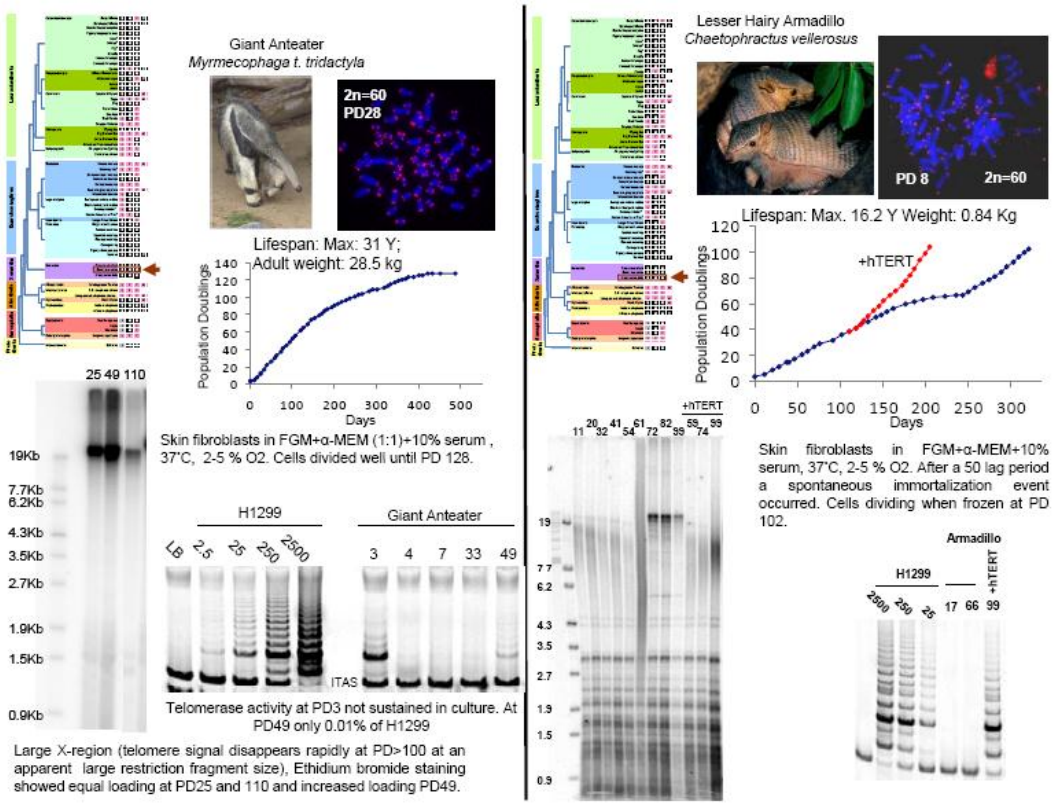

Mexican Free-tailed Bat
Tadarida brasilensis
2n=48
PD 7
Lifespan: Wild > 8 Y; Max. 12 Y;
Weight: 0.0125 Kg
Population Doublings
Days
African Pigmy Hedgehog
Atelerix albiventris
PD15
2n=48
Lifespan: Av. Wild: 2-3 Y Max cap.: 11.4 Y
Adult weight: 0.600 kg
+E6/E7
stasis
Population Doublings
Days
PD6
48
30
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
0.9
1% agarose FIGE gel
Skin fibroblasts grown in DMEM+10%serum, 37˚C, 2-5 % O2, still dividing well when frozen at PD 105.
H1299
MFTB
LB
2500
250
25
6
38
17 35 47 60 74 89 104 118 132
12 29 41 54 66 81 96 111 126
48
29
19
7.7
4.3
2.7
1.5
Skin fibroblasts in FGM+α-MEM (1:1)+10%serum, 37˚C, 2-5 % O2. Stasis at PD 6. After introduction of E6E7 cells divided well when until frozen at PD 133.
H1299
Hedgehog
LB
25
250
2500
23
13
1% agarose 5-50 kb resolution FIGE gel.

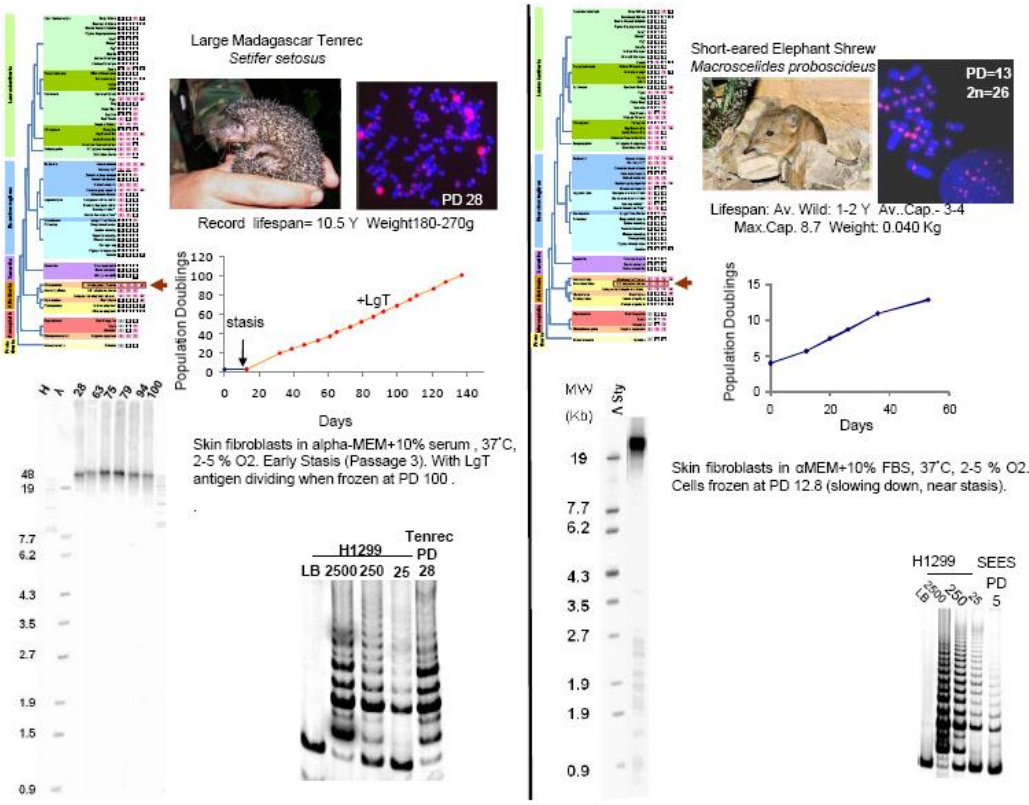

Soricidae sp. Shrew
Probably Notiosorex crawfordi
2n=68
PD13
Lifespan: Av. 12-13 months Weight: 0.004 Kg
Population Doublings
probable
stasis
Days
Skin fibroblasts in FGM+α-MEM+10% serum, 37°C, 2-5 % O2. Cells dividing well until frozen at PD 75. The initial prolonged slow growth phase may represent stasis with escape as often seen in laboratory mouse cultures.
H1299
Lake Shrew
LB
2500
250
25
17 23 34 57
9
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
0.9
Sonoran Deer Mouse
Peromyscus maniculatus sonoriensis
Lifespan: Av. Wild: <2 Y; Max.
Captivity 8.3 Y Adult Weight: 20.5 g
Population Doublings
+E6/E7
stasis
Days
Kidney fibroblasts in D-MEM+10% serum, 37°C, 2-5 % O2. Cells dividing after introduction of E6E7 until frozen at PD 109.
MW
(kb)
19
7.7
6.2
4.3
2.7
1.9
H1299
Deer Mouse
LB
25
250
2500
15 29 43 54

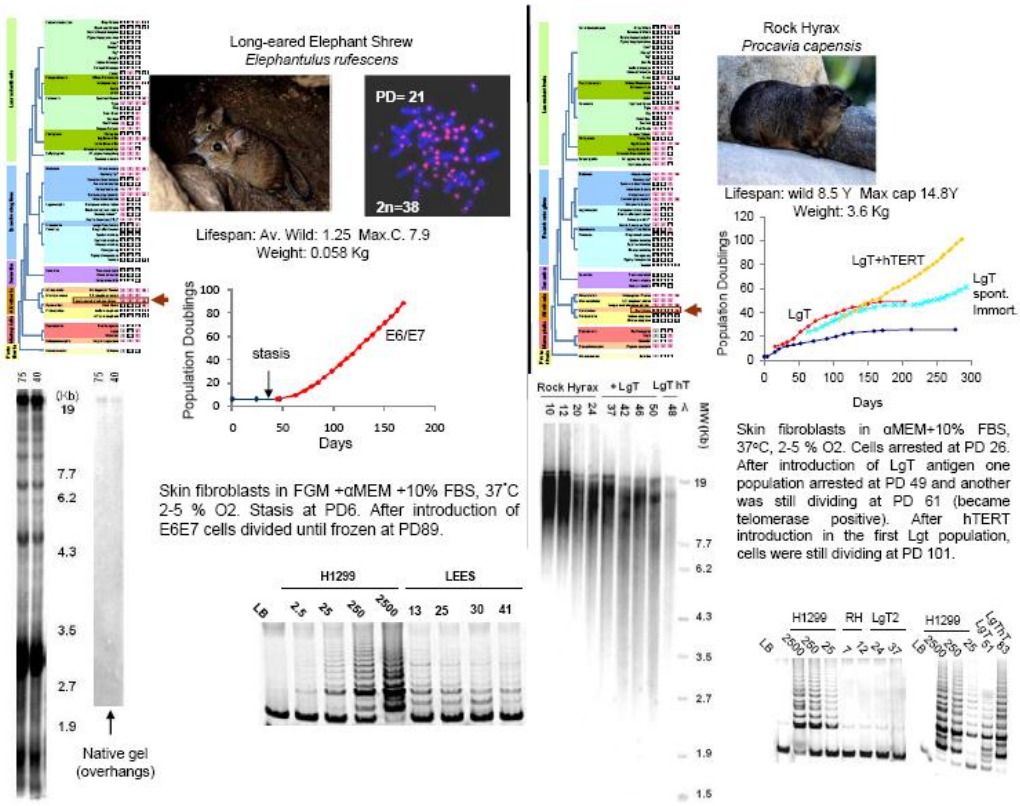

American Beaver
Castor canadensis
Lifespan: Av. Wild: 10-20 Y Max. 23.4 Y
Weight: 20.250 Kg
Population Doublings
+LgT
probable
stasis
Days
Skin fibroblasts in D-MEM+10% serum, 37°C , 2-5 % O2. Cells grew well until growth arresting at PD 29. After addition of SV40 LgT antigen, cells divided until clonal growth was observed at PD at PD 32. The initial prolonged slow growth phase may represent stasis with escape as often seen in laboratory mouse cultures.
H1299
Beaver
LgT
No LgT
LB
Telomerase positive after PD 23 even in absence of LgT
Naked Mole Rat
Heterocephalus glaber
2n=60
PD4
Lifespan in the Wild: non-breeders: 4 Y ; breeders: 17 Y. Max. captivity: 30Y.
Weight: 0.035 Kg
+E6/E7
Stasis
Kidney fibroblasts in D-MEM+10% serum, 32°C, 2-5 % O2. Stasis at PD 4. After introduction of E6E7 cells were dividing well when frozen at PD 153.
PD 30
PD 151

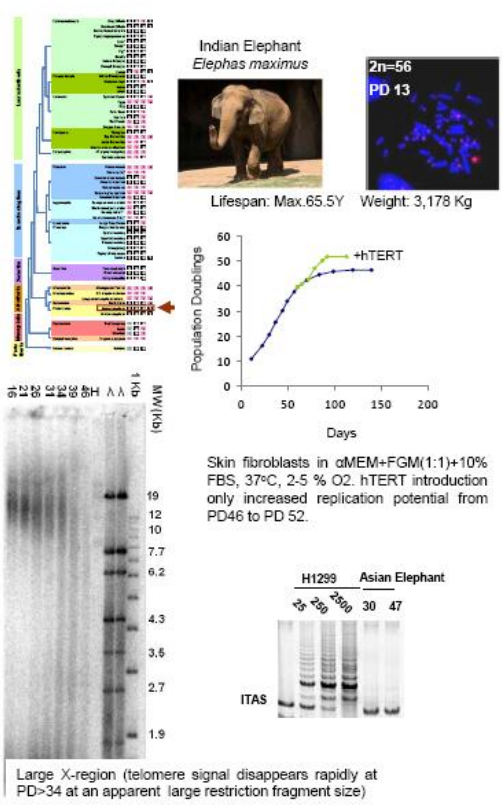

Eastern Grey Squirrel
Sciuris carolinensis
Lifespan: Av. Wild: 2-3 Y Max Capt: 23.6 Y Adult
Weight: 0.553 Kg
Population Doublings
+E6/E7
Stasis
Days
Skin fibroblasts in D-MEM+10% serum, 37°C, 2-5 % O2. Cells entered stasis at PD 1 and were dividing well after introduction of E6E7 until frozen at PD 110.
9 15 19 22 24 28 32 36 40 44 48 76 81 87 93 98 104 110
19
7.7
6.2
4.3
ITAS
2.7
H1299
Squirrel
LB 25 250 2500 15 22 32
MW (kb)
Y H MB
48
30
19
7.7
4.3
0.9
FIGE gel
Mountain Beaver
Aplodontia rufa
2n=46
PD4
Lifespan: Av. Wild: >6Y Max. Wild 10Y?
Weight: 1.125 Kg
LgT
Days
Skin fibroblasts in D-MEM+10% serum , 37°C , 2-5 % O2. Cells divided well until frozen at PD 49.
H1299
LB 2500 250 25 PD 5

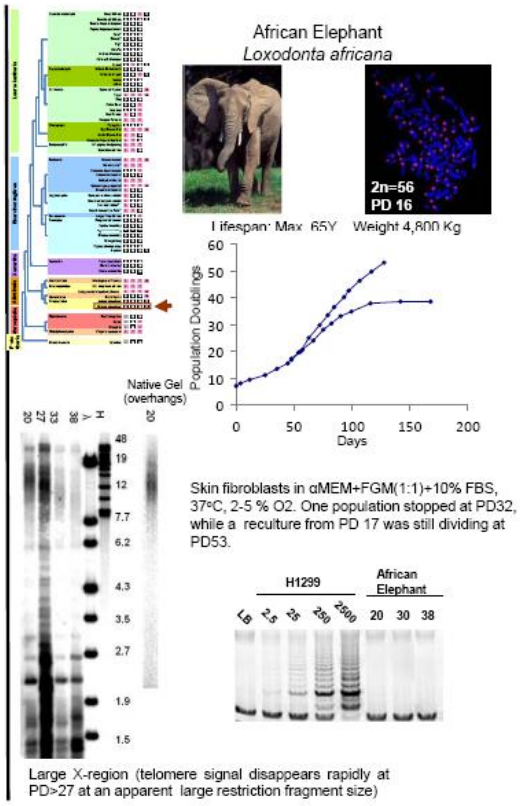

Large Tree Shrew
Tupaia tana
PD 12
2n=60
Lifespan: 11.6 Y
Weight: 0.198 Kg
LgT
Stasis
Skin fibroblasts in αMEM+10% FBS, 37°C, 2-5 % O2. STASIS at PD 5. Adenoviral Sv40LgT antigen (AdLgtT) followed by retroviral LgT (RLgT), cells dividing when frozen at PD 111.
H1299
LTS
AdLgt
RLgt
LB
250
2500
5
15
24
42
ITAS
Hoffmann's Two Toed Sloth
Choloepus hoffmani
Lifespan: Average wild: 20 Y; Max. Cap: 35 Y
Weight: 6.25 Kg
Population Doublings
Days
Skin fibroblasts in FGM+α-MEM+10% serum, 37°C 2-5 % O2. Cells dividing when frozen at PD 13.
H1299
LB
2500
250
25
PD 7

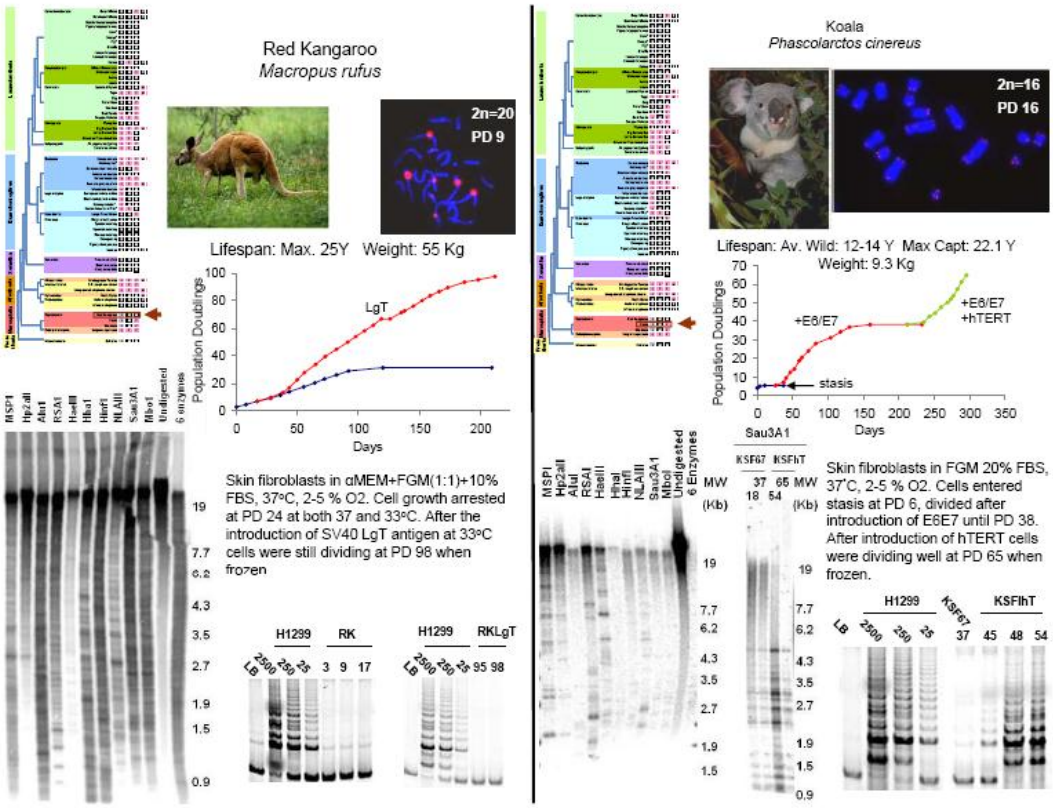

Giant Anteater
Myrmecophaga t. tridactyla
2n=60
PD28
Lifespan: Max: 31 Y;
Adult weight: 28.5 kg
Population Doublings
Days
Skin fibroblasts in FGM+α-MEM (1:1)+10% serum , 37°C, 2-5 % O2. Cells divided well until PD 128.
25 49 110
19Kb
7.7Kb
6.2Kb
4.3Kb
3.5Kb
2.7Kb
1.9Kb
1.5Kb
0.9Kb
H1299
Giant Anteater
LB 2.5 25 250 2500
3 4 7 33 49
ITAS
Telomerase activity at PD3 not sustained in culture. At PD49 only 0.01% of H1299
Large X-region (telomere signal disappears rapidly at PD>100 at an apparent large restriction fragment size), Ethidium bromide staining showed equal loading at PD25 and 110 and increased loading PD49.
Lesser Hairy Armadillo
Chaetophractus vellerosus
PD 8
2n=60
Lifespan: Max. 16.2 Y Weight: 0.84 Kg
+hTERT
Population Doublings
Days
Skin fibroblasts in FGM+α-MEM+10% serum, 37°C, 2-5 % O2. After a 50 lag period a spontaneous immortalization event occurred. Cells dividing when frozen at PD 102.
+hTERT
11 20 32 41 54 61 72 82 95 55 74 99
19
7.7
6.2
4.3
3.5
2.7
1.9
1.5
0.9
Armadillo
H1299
+hTERT
2500 250 25 17 66 99

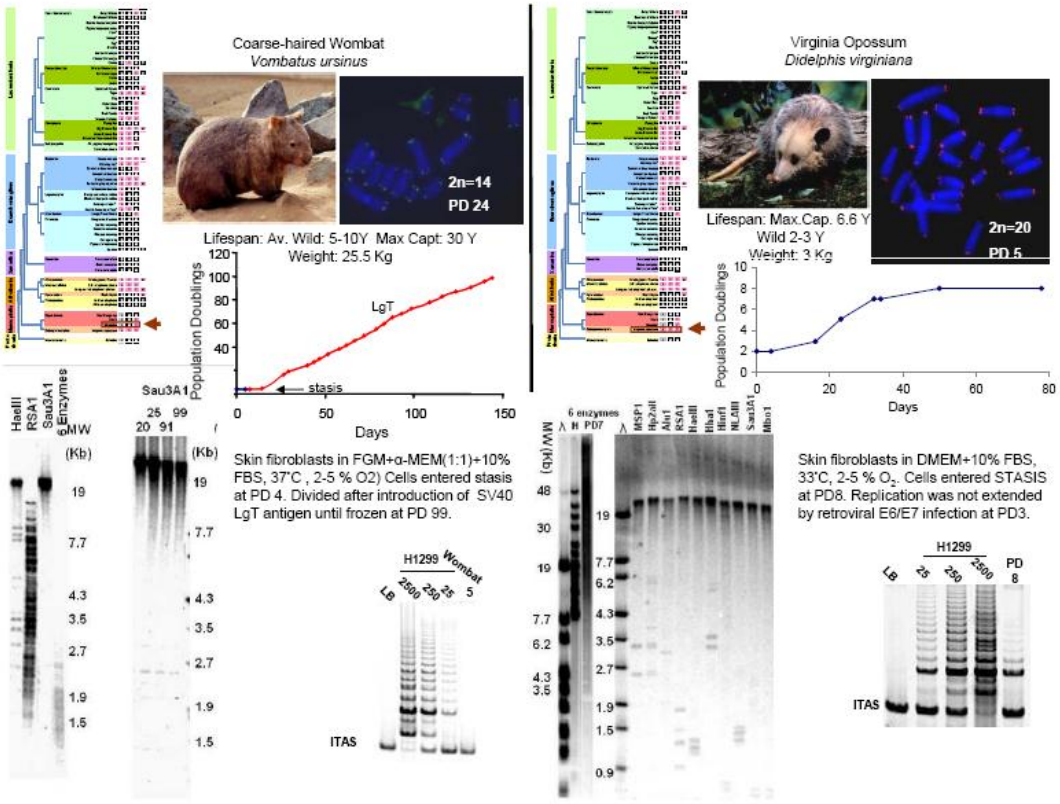

Large Madagascar Tenrec
Setifer setosus
PD 28
Record lifespan= 10.5 Y Weight180-270g
Population Doublings
stasis
+LgT
Days
Skin fibroblasts in alpha-MEM+10% serum , 37˚C, 2-5 % O2. Early Stasis (Passage 3). With LgT antigen dividing when frozen at PD 100 .
H1299
Tenrec PD
LB 2500 250 25 28
Short-eared Elephant Shrew
Macroscelides proboscideus
PD=13
2n=26
Lifespan: Av. Wild: 1-2 Y Av..Cap.- 3-4
Max.Cap. 8.7 Weight: 0.040 Kg
Population Doublings
Days
Skin fibroblasts in αMEM+10% FBS, 37˚C, 2-5 % O2. Cells frozen at PD 12.8 (slowing down, near stasis).
MW (Kb)
λ Sty
H1299
SEES PD
LB 2500 250 25 5

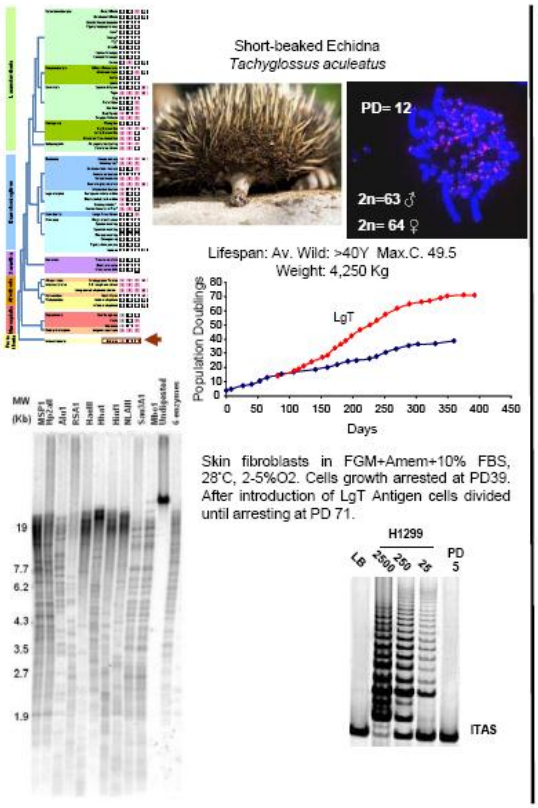

Long-eared Elephant Shrew
Elephantulus rufescens
PD= 21
2n=38
Lifespan: Av. Wild: 1.25 Max.C. 7.9
Weight: 0.058 Kg
Population Doublings
stasis
E6/E7
Days
Skin fibroblasts in FGM +αMEM +10% FBS, 37°C 2-5 % O2. Stasis at PD6. After introduction of E6E7 cells divided until frozen at PD89.
H1299
LEES
LB
2.5
25
250
2500
13
25
30
41
(Kb)
19
7.7
6.2
4.3
3.5
2.7
1.9
Native gel
(overhangs)
Rock Hyrax
Procavia capensis
Lifespan: wild 8.5 Y Max cap 14.8Y
Weight: 3.6 Kg
LgT+hTERT
LgT
LgT spont. Immort.
Skin fibroblasts in αMEM+10% FBS, 37°C, 2-5 % O2. Cells arrested at PD 26. After introduction of LgT antigen one population arrested at PD 49 and another was still dividing at PD 61 (became telomerase positive). After hTERT introduction in the first Lgt population, cells were still dividing at PD 101.
Rock Hyrax
+LgT
LgT hT
MW(kb)
RH
LgT2
LgTT2


Indian Elephant
Elephas maximus
2n=56
PD 13
Lifespan: Max.65.5Y
Weight: 3,178 Kg
+hTERT
Population Doublings
Days
Skin fibroblasts in αMEM+FGM(1:1)+10% FBS, 37ºC, 2-5 % O2. hTERT introduction only increased replication potential from PD46 to PD 52.
H1299
Asian Elephant
ITAS
Large X-region (telomere signal disappears rapidly at PD>34 at an apparent large restriction fragment size)


African Elephant
Loxodonta africana
2n=56
PD 16
Lifespan: Max. 65Y
Weight 4,800 Kg
Native Gel
(overhangs)
Population Doublings
Days
Skin fibroblasts in αMEM+FGM(1:1)+10% FBS, 37ºC, 2-5 % O2. One population stopped at PD32, while a reculture from PD 17 was still dividing at PD53.
H1299
African Elephant
Large X-region (telomere signal disappears rapidly at PD>27 at an apparent large restriction fragment size)


Red Kangaroo
Macropus rufus
2n=20
PD 9
Lifespan: Max. 25Y Weight: 55 Kg
Population Doublings
Days
LgT
Skin fibroblasts in αMEM+FGM(1:1)+10% FBS, 37°C, 2-5 % O2. Cell growth arrested at PD 24 at both 37 and 33°C. After the introduction of SV40 LgT antigen at 33°C cells were still dividing at PD 98 when frozen
MSPI
Hpa2all
Alul
RSAI
HaeIII
Hhal
HinfI
NLAIII
Sau3A1
NlaI
Undigested
6 enzymes
MW (Kb)
H1299
RK
RKLgT
LB
2500
250
25
3
9
17
95
98
Koala
Phascolarctos cinereus
2n=16
PD 16
Lifespan: Av. Wild: 12-14 Y Max Capt: 22.1 Y
Weight: 9.3 Kg
+E6/E7
+E6/E7
+hTERT
stasis
Sau3A1
KSF67
KSFIhT
18
54
65
37
45
48
Skin fibroblasts in FGM 20% FBS, 37°C, 2-5 % O2. Cells entered stasis at PD 6, divided after introduction of E6E7 until PD 38. After introduction of hTERT cells were dividing well at PD 65 when frozen.


Coarse-haired Wombat
Vombatus ursinus
2n=14
PD 24
Lifespan: Av. Wild: 5-10Y Max Capt: 30 Y
Weight: 25.5 Kg
Population Doublings
LgT
stasis
Days
Virginia Opossum
Didelphis virginiana
2n=20
PD 5
Lifespan: Max.Cap. 6.6 Y
Wild 2-3 Y
Weight: 3 Kg
Population Doublings
Days
HaeIII
RSA1
Sau3A1
6 Enzymes
MW
(Kb)
Sau3A1
20 91 25 99
Skin fibroblasts in FGM+α-MEM(1:1)+10% FBS, 37°C , 2-5 % O2) Cells entered stasis at PD 4. Divided after introduction of SV40 LgT antigen until frozen at PD 99.
H1299
Wombat
LB
2500
250
25
5
ITAS
6 enzymes
MW (kb)
MSP1
Hpa2all
Alu1
RSA1
HaeIII
Hha1
Hin1
NLAIII
Sau3A1
Mbo1
Skin fibroblasts in DMEM+10% FBS, 33°C, 2-5 % O2. Cells entered STASIS at PD8. Replication was not extended by retroviral E6/E7 infection at PD3.
H1299
LB
25
250
2500
PD 8
ITAS


Short-beaked Echidna
Tachyglossus aculeatus
PD= 12
2n=63 ♂
2n= 64 ♀
Lifespan: Av. Wild: >40Y Max.C. 49.5
Weight: 4,250 Kg
Population Doublings
LgT
Days
Skin fibroblasts in FGM+Amem+10% FBS, 28°C, 2-5%O2. Cells growth arrested at PD39. After introduction of LgT Antigen cells divided until arresting at PD 71.
H1299
LB
2500
250
25
PD 5
ITAS
MW (Kb)
19
7.7
6.2
4.3
3.5
2.7
1.9